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(54) Title: ASSAYS, DEVICES AND KITS FOR DETERMINING MALE FERTILITY

(57) Abstract

Assays, devices and kits for identifying sperm samples with high pregnancy potential (e.g. for use in an assisted reproductive technology) or sperm samples with low pregnancy potential (e.g. for identifying potentially infertile males or for evaluating the effectiveness of a male contraception means) are disclosed.

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ASSAYS, DEVICES AND KITS FOR DETERMINING MALE FERTILITY

Background of the Invention

Determining the Fertility of a Sperm Sample

According to recent studies, male infertility is responsible almost 40% of the time that a couple is unable to conceive a child. In addition, use of male contraceptives is on the rise. For example, according to current estimates, more than 500,000 vasectomies are performed in the U.S. each year and about 2,000,000 are performed worldwide. In addition to vasectomies, a variety of oral contraceptives for use by males are in development. Although male contraceptives decrease the probability that a fertile male will initiate a pregnancy, no male contraceptive is 100% effective. Further, most male contraceptives require a certain period of time in which to take effect.

For a semen sample to be considered fertile according to standards set by the World Health Organization, at least two 1.5-5.0 milliliter ejaculate volumes obtained from a male must contain a sperm density of greater than 20 million spermatozoa/mL and/or a percent motility of 60% with a forward progression greater than 2 (on a 1-4 scale). In addition, the semen samples should show no evidence of sperm agglutination, pyospermia or hyperviscosity (Sigman, M., et al., Evaluation of the subfertile male. In: Lipschultz, LI and SS Howards eds. *Infertility in the Male*, 2nd ed. Chicago: Mosby-Year Book, 1991; p.184).

By convention, male infertility is diagnosed based on low sperm motility and/or count. However, motility analyses can produce false negatives, since viable sperm may appear non-motile due to damage sustained during processing. With regard to sperm count, 20 million spermatozoa/mL or greater is generally considered to be in the fertile range. However, non-motile, non-viable sperm can be included in the count. Sperm count and motility are typically assessed using commercially available instruments, such as light microscopes and computerized videoanalysis systems.

U.S. Patent No. 5,068,089 describes a home kit for testing fertility of human sperm based on ability of the sperm to reduce a dye. The extent of reduction (displayed colorimetrically), is said to be indicative of sperm fertilizing ability. However, this test is

time consuming, requires incubation at a temperature above room temperature and does not distinguish between reduction due to sperm cells or other cells, which may be present in a semen sample.

U.S. Patent No. 5,219,729 describes a laboratory assay for determining the fertilizing ability of sperm based on the affinity of binding to an oocyte zona pellucida fragment. The tighter the binding, the greater the fertilizing ability of the sperm sample. However, this assay requires freshly prepared oocyte fragments and at least four hour's time during which the sperm must be kept in contact with the oocyte fragment.

U.S. Patent No. 5,434,057 describes assays and kits for determing male fertility based on the detection of fumarase activity. However, since fumarase is ubiquitously present in cells including cells that may be present in a sperm sample (epithelial, leukocyte and bacterial or fungal), whether fumarase activity is an accurate indicator of sperm count and motility, as claimed in the patent, is dubious.

A simple, rapid and accurate assay for determining the fertility of a sperm sample in a reference laboratory, doctor's office or at home is needed.

Increasing the Effectiveness of an Assisted Reproductive Technology

Assisted reproductive technologies (ARTs), such as *in vitro* fertilization (IVF), gamete intrafallopian transfer (GIFT), intrauterine insemination (IUI) and sperm intracytoplasmic injection (ICSI) offer ways to initiate a pregnancy when natural approaches have been unsuccessful. These techniques are also useful for breeding animals or producing transgenic animals.

However, ARTs do not always result in a successful pregnancy. For example, IVF has an estimated success rate of about 25%, while GIFT is estimated as being successful in about 31% of attempts. One factor, which may contribute to an unsuccessful ART attempt is that not all sperm samples are capable of fertilization. According to recent studies, male infertility is responsible almost 40% of the time that a couple is unable to conceive a child.

In addition, the chance that a sperm sample will be incapable of initiating a pregnancy is increased when that sample has been stored for any period of time or cryopreserved. This finding has particular significance in view of the use of cryopreserved sperm in ARTs. Cryopreservation can result in sublethal cryodamage, in which cell viability post-thaw is lost more rapidly at later times than in fresh cells. Sublethal cryodamage has been shown to be due in part to membrane embrittlement during the phase transitions involved in freezing and thawing (Alvarez, J.G. and B.T. Storey (1993) *J. Androl.* *14* (3):

199-209). To a lesser degree, sublethal cryodamage has been shown to be caused by spontaneous lipid peroxidation (SLP) of sperm phospholipids (Alvarez, J.G. and B.T. Storey (1993) *J. Androl. 14* (3): 199-209 and J.G. Alvarez and B.T. Storey (1992) *J. Androl 13(3)*: 232-241). Spontaneous lipid peroxidation appears to be the major factor limiting the motile lifetime of sperm that has not been cryopreserved (Alvarez, J.G. and B.T. Storey (1988) *Gamet Res. 23*:77-90; and Alvarez, J.G. and B.T. Storey (1985) *Biol. Reprod. 32*: 342-351). The selective oxidation of phospholipid-bound polyunsaturated fatty acid moieties resulting from spontaneous lipid peroxidation leads to extensive oxidative damage to the sperm plasma and acrosomal membranes and DNA.

However, cryopreservation of sperm samples is routinely performed in ART procedures and in fact is required in order to test the donor for the presence of transmissible infectious agents (e.g., HIV) prior to insemination. For example, a donor is typically tested six months after producing a particular sample and only if the test is negative will the stored sample be used for insemination.

More and more couples are turning to ARTs to conceive a child. In addition, in vitro fertilization technologies are increasingly being used by breeders of livestock and in generating transgenic animals. A method for increasing the success rate of ARTs is needed.

Summary of the Invention

In general, the invention features devices, procedures and kits for isolating and quantitating motile sperm from a sperm-containing sample (e.g. semen) and/or for determining the pregnancy potential of a sperm sample. In general, the devices comprise a container for isolating a sperm containing sample, which is preferably adapted to separate sperm based on motility. The container can optionally be conformed to facilitate collection (e.g. funnel-shaped). In a preferred embodiment, a retainer for accomodating entry and migration of motile sperm, but not non-motile cells in the sperm containing sample, is positioned inside of the container. Preferably at an appropriate site within the retainer is positioned a means to determine the pregnancy potential of sperm that migrate into the retainer. Alternatively, isolated motile sperm can be obtained from the retainer and separately tested.

In one embodiment, the retainer is a compartment (e.g. a tube), which is in fluid communication with the sperm containing sample. In another embodiment, the retainer is a fluid, which is less dense than the sperm containing sample and thereby facilitates passage of motile sperm, but not non-motile cells. In a further embodiment, the retainer includes a porous membrane, which separates the sperm containing sample from an isolation area and which thereby prevents passage of non-motile sperm or other cells that may be

present in the sperm containing sample, but allows passage of motile sperm into the isolation area.

In a second aspect, the invention features assays for identifying sperm samples with high pregnancy potential. The assays can be used to provide an indication of the fertility status of the male donor or can be selected for use in an Artificial Reproductive Technology (ART). In a preferred embodiment, the process comprises the steps of: i) obtaining multiple sperm samples from a donor over time, ii) obtaining an aliquot from each sperm sample, iii) testing each aliquot to determine pregnancy potential, and iv) using at least one sperm sample having high pregnancy potential in an ART to initiate a pregnancy. In a preferred embodiment, the testing is based on quantitating an indicator of lipid peroxidation or a change in an indicator induced by a stress. A particularly preferred indicator of sperm lipid peroxidation is sperm superoxide dismutase (SOD) activity, which can be quantitated, for example, using an anti-SOD antibody.

In a third aspect, the invention features screening assays for detecting the pregnancy potential of sperm samples and thereby identifying potentially infertile males or evaluating males following vasectomy or once a particular contraceptive has been administered. In a preferred embodiment, the screening assay is based on determining motile sperm count. In one embodiment, motile sperm are first isolated and counted. Quantitation of less than about 50,000 motile spermatozoa/mL indicates that the contraceptive has been effective and that the sperm sample has low pregnancy potential. In another embodiment, quantitation of sperm is performed directly on the sperm containing sample (without separation of motile and immotile sperm) and quantitation of less than about 20 million spermatozoa/mL indicates that the sample has low pregnancy potential; greater than about 20 million spermatozoa/mL and less than about 40 million spermatozoa/mL indicates that the sample has borderline pregnancy potential; and greater than about 40 million spermatozoa/mL indicates that the sample has high pregnancy potential. The cutoff value of 20 million spermatozoa/mL includes both motile and immotile spermatozoa. Although this value is not diagnostic of infertility, it is intended to be used as part of a screening test. In this way, males that use the kit to test their fertility potential and have less than about 20 million spermatozoa/mL will be alerted to a potential infertility problem.

In a fourth aspect, the invention features sperm diagnostic kits or systems which comprise a number of simple reagents and devices packaged in a box. In one embodiment for use in identifying a high pregnancy potential sperm sample, the kit can include a sperm isolation means and a means for identifying a high pregnancy potential sperm sample. Optionally, the sperm isolation means is of an appropriate conformation (e.g. funnel shaped) to facilitate collection. Also optionally, a reagent for liquefying semen (e.g.

pronase or chymotrypsin) is included in the kit for use in sperm isolation. Preferably the means for identifying sperm with high pregnancy potential is based on lipid peroxidation and the means for screening for pregnancy potential (e.g. infertility or effective male contraception) is based on sperm count.

The instant disclosed assays, devices and kits are easy to perform and can be completed in less than about 15 minutes' time. The assays, devices and kits provide information which is useful, for example, in monitoring the impact of changes in such factors as diet, sleep, exercise, exposure to smoke or other carcinogens, and intake of alcohol or drugs on the fertility of sperm samples produced thereafter. Also, the devices, kits and methods can indicate whether a particular male fertility or contraceptive treatment has been effective or whether sperm subsequently obtained from the same male will initiate a pregnancy upon contact with an oocyte. In addition, the assays, devices and kits are useful for determining whether a particular sperm sample is suitable for use in an ART.

Certain of the assays, devices and kits are appropriate for use in a reference laboratory, while others can be used in a doctor's office and/or at home. Other features and advantages will become readily apparent from the following Detailed Description and Claims.

Brief Description of the Figures

Figure 1 is a perspective view of one embodiment of a sperm isolation device, which includes a tube for receiving and capturing motile sperm.

Figure 2 is a perspective view of another embodiment of a sperm isolation device, which includes a less dense fluid layer in contact with a more dense sperm-containing sample.

Figure 3 is a perspective view of yet another embodiment of a sperm isolation device, in which a porous membrane separates the sperm sample from a motile sperm collection area.

Figure 4 is a graph plotting the motility recovery after cryopreservation for sperm samples having a stress test score of greater than about 0.8 and samples having a stress test score of less than about 0.8.

Detailed Description

The invention pertains to easy to use devices that can rapidly recover motile sperm from a sperm containing sample (e.g. semen) and assays and kits, which employ the devices for use in identifying a high pregnancy potential sperm sample. The invention also pertains to assays and kits for screening males for infertility and for confirming whether a male contraceptive is effective or has taken effect.

Preferred sperm isolation devices are based on sperm motility and comprise a housing means for holding sperm, an isolation means selectively disposable within the housing means for isolating motile sperm and a means for testing the motile sperm to determine pregnancy potential. One embodiment is illustrated in Figure 1. A container 10 that has side walls 12, 14, 16 and 18 and a bottom portion 20 forming a sample well 21 for receiving a sample 30 containing ejaculate from a male donor. Although illustrated as cubical, the container 10 can have any selected shape or size suitable for the capture and retention of a sperm containing sample. A retainer 24 can be positioned within the sample well 21 of the container, and is preferably placed in fluid communication with the sample 30. Those of ordinary skill will recognize that the retainer 24 can be mounted to the floor portion 20 of the container 10 by a number of known methods, including the use of an adhesive, or can be supported within the container 10 by any suitable support means. Although illustrated as cylindrical, the retainer 24 can have any selected shape or size suitable for the isolation and retention of motile sperm, and can include commercially available instruments, such as tubes, capillaries, straws, pipettes, and other suitable receptacles having an internal conduit. Additionally, the container 10, although illustrated as having a substantially rectangular shape, can be configured to have any selected shape, such as a funnel-like configuration, to assist the male donor in collecting and capturing a sperm sample. The container 10 and retainer 24 can be made from any suitable biocompatible material, such as glass or plastic.

The illustrated sample 30 can contain both motile and non-motile sperm. According to a preferred practice, the retainer 24 is positioned within the well 21 or constructed to allow the motile sperm to migrate into the conduit 22 of the receptacle 24. The receptacle 24 captures the motile sperm, which are then analyzed to determine the pregnancy potential of the sperm sample. For example, after a sufficient period of time has elapsed to ensure that motile sperm present in the sample 30 have migrated into the retainer 24, the retainer can be removed from the container 10 and placed within a second container 31, e.g., a test tube, containing a means for testing the motile sperm to determine the pregnancy potential of the sample 34.

Alternatively, a detection mechanism 36 can be mounted within the conduit 22 of the retainer 24. The motile sperm that migrate into the conduit 22 preferably contact the detection mechanism 36. The detection mechanism preferably includes a suitable membrane or substrate containing a means for testing motile sperm to determine the pregnancy potential of the sperm containing sample.

Motile sperm can alternatively be isolated from non-motile cells in a sperm sample based on contact with a mechanical or fluid barrier or gradient. For example, as shown in Figure 2, a relatively dense sperm-containing sample can be contacted with a less dense fluid layer, which is preferably of a similar temperature, pH and salt concentration as the sperm-containing sample. In Figure 2 and in all subsequent figures, like parts are represented throughout with the same reference numerals plus a superscript prime. The illustrated container 10' includes a first fluid layer 40 and a second fluid layer 46. The first fluid layer 40 includes a sperm sample from the male donor and optionally an appropriate reagent for liquefying the semen sample, e.g. pronase or chymotrypsin. The layer 40 typically includes motile and/or non-motile sperm. The second fluid layer 46 is preferably a fluid having a density that is less than the density of the first fluid layer 40, thus creating a density gradient along the height of the container 10'. The decreasing density between layers 40 and 46 promote the migration of motile sperm from the sample fluid layer 40 into the collection fluid layer 46. This axial layered construction of the fluids 40 and 46 can be accomplished by selecting appropriate fluids that are emissible or partially emissible relative to one another.

According to a preferred practice, a sperm sample is placed within the container or is mixed with a reagent for liquefying ejaculate prior to introduction into the container 10'. A second fluid layer 46 having a lower density is then introduced into the container 10' and is separated from and disposed axially above the first fluid layer 40. After a sufficient amount of time, the motile sperm present in the sample layer 40 migrate into the second fluid layer 46 in response to the diminishing density gradient along the height of the container 10'. Thus, the motile sperm are isolated in the second fluid layer 46. After a selected period of time (e.g., five minutes) a sample of fluid from fluid layer 46 is removed, as by a pipette 50, and is introduced into a test tube 52 which holds a test solution 54. The solution preferably contains a hypotonic solution having peroxidase-conjugated anti-SOD and/or anti-GPx IgG polyclonal antibodies. A test strip containing bound sheep anti-SOD and/or anti-GPx IgG polyclonal antibodies is placed into the test solution, removed and then placed in a final solution containing a peroxidase substrate (e.g. hydrogen peroxide and DAB). In response to the presence of motile sperm, the test strip changes to a selected color. This color is matched to a color chart to determine the pregnancy potential of the sperm

sample. Alternatively, the pipette containing motile sperm can be directly applied to a test strip, which contains a sperm reagent.

According to an alternate embodiment, as illustrated in Figure 3, the second collection fluid layer 46 can be replaced with a porous membrane 60. The permeable porous membrane 60 introduces or presents a mechanical resistance to the migration of non-motile sperm from the sample layer 40' into or through the membrane 60. However, the porous membrane 60 facilitates the migration and hence the isolation of motile sperm in the isolation layer 10' by allowing motile sperm to pass between sample layer 40' and the membrane 60. The porous membrane 60 can be composed of any suitable biocompatible material that facilitates the passage of motile sperm without introducing damage thereto.

In another aspect, the invention relates to preferred methods for determining the pregnancy potential of a sperm sample. A sperm sample with "high pregnancy potential" as described herein has a greater than about 50% probability for initiating a pregnancy upon contact with an oocyte. Although a sperm sample having high pregnancy potential has an increased probability of initiating a pregnancy, contact of a high pregnancy potential sperm sample with an oocyte does not guarantee a successful pregnancy. Other factors, such as inability of an oocyte to decondense human sperm chromatin, defective oocyte DNA (e.g. due to age), two-cell embryo block or early embryo demise, may prevent the initiation of a pregnancy even by a high pregnancy potential sperm sample.

A sperm sample with "low pregnancy potential", on the other hand, has very little or no chance of initiating a pregnancy upon contact with an oocyte. Indication that a sperm sample provided by a particular male has low pregnancy potential does not indicate that subsequent sperm samples will also have low potential. Since the pregnancy potential of the sperm samples produced by a given male can change over time and can be influenced by such factors as diet, sleep, exercise, exposure to smoke or other carcinogens, or intake of alcohol or drugs, the pregnancy potential of a particular sperm sample is in general only accurate for a period of about 48 hours.

Preferred sperm containing samples (e.g. semen) for use in the disclosed assays are obtained from a human or animal (e.g. a bull, stallion, ram or other domesticated animal or an endangered animal). To ensure accuracy, tests are preferably performed on freshly collected ejaculate.

Preferred tests for identifying sperm samples with high pregnancy potential are lipid peroxidation tests, which measure an indicator of lipid peroxidation or a change in an indicator over a period of time under defined conditions. The value obtained is then

compared with a standard value for that particular indicator to determine the pregnancy potential of that sperm sample.

A stress test measures a change in a particular indicator of lipid peroxidation over a period of time in response to a stress (a stress test). A stress test score can be obtained by dividing a measured post-stress value of an indicator of lipid peroxidation by a measured pre-stress value. Examples of appropriate stresses or stressing agents for obtaining a test score include radiation (such as thermal (e.g. as described in Example 2), electromagnetic), freeze-thawing, oxidation or exposure to chemical agents (e.g. oxidizing agents such as the ferrous iron/ascorbate system described in Example 3).

Examples of lipid peroxidation indicators that change in response to a stress include:

Motility: When motility is used as an indicator of lipid peroxidation and the stress is thermal (e.g. incubation for at least about 1 hour at a temperature in the range of about 27-45^{o}C), a test score of greater than about 0.8 indicates high pregnancy potential. A test score of less than about 0.8 indicates low potential.

Lipid peroxidation breakdown products: Samples that have a relatively high post-stress value of lipid peroxidation breakdown products (such as lipid hydroperoxides, malonaldehyde, pentane and ethane) relative to pre-stress value (test score < 0.5) have a low probability of resulting in a successful pregnancy, while test scores of about 0.5 or greater increase the probability of initiating a pregnancy. The lipids can be analyzed spectrophotometrically. For example, lipid hydroperoxides can be extracted with hexane and detected at 233nm. Malonaldehyde can be measured at 532nm following reaction with the thiobarbituric acid (Tapel, A.L. et al., (1959) *Arch Biochem Biophys. 80*:326). Pentane and ethane can be measured by head-space gas chromatography.

Ratio of membrane phospholipids: Samples that oxidize at high rates have phosphatidylethanolamine (PE)/ phosphatidylcholine (PC) ratios < about 0.5, while samples that oxidize at low rates (test scores > about 0.8 as indicated by loss of motility) have PE/PC ratios of greater than about 0.7. Therefore, sperm with high pregnancy potential have PE/PC ratios greater than about 0.7, but less than about 1.5, sperm with some pregnancy potential have PE/PC ratios of greater than about 0.5 and less than about 0.7, and sperm

with low pregnancy potential have PE/PC ratios of less than about 0.5. PE and PC can be measured, for example, by high-performance thin-layer chromatography (Alvarez, J.G. et al., (1987) *J Liquid Chromatogr 10: 355*).

Table I
Pregnancy Potential Based on Test Score

Indicator	Stress	Test Score
Lipid peroxidation breakdown products (e.g. lipid hydroperoxides, malonadehyde, pentane, ethane)	oxidation, increased temperature	≥ 0.5 High Pregnancy Potential <0.5 Low Pregnancy Potential
PE/PC	oxidation	>0.7 - <1.5 = High Pregnancy Potential >0.5 - 0.7 = Some Pregnancy Potential <0.5 = Low Pregnancy Potential
Motility	increased temperature	≥ 0.8 = High Pregnancy Potential < 0.8 = Low Pregnancy Potential

Examples of lipid peroxidation indicators that can be directly measured as an indication of pregnancy potential include:

Oxidation of DNA: Samples that oxidize at high rates have higher levels of oxo-8-deoxyguanosine (oxo^{8}dG), which can be measured, for example, by high-pressure liquid chromatography (HPLC) using electrochemical detection (Fraga, C.G. et al., (1991) *Proc Natl Acad Sci 88*:11003.). Sperm with high pregnancy potential have less than about 20 fmol of oxo^{8}dG/ µg of DNA, while sperm with low pregnancy potential have greater than about 40 fmol of oxo^{8}dG/ µg of DNA.

Superoxide dismutase (SOD) activity: Solid phase-bound anti Cu/Zn-SOD antibodies and enzyme labeled anti-Cu/Zn-SOD antibodies can be used to detect SOD activity as described in Example 3. Sperm samples with SOD activities greater than or equal to about 9U/10^8 cells have been shown to be associated with high pregnancy rates after IVF (i.e. have high pregnancy potential), while activities less than about 7U/10^8 cells are associated with low pregnancy rates (i.e. low pregnancy potential).

Surface SOD immunofluorescence Samples that oxidize at high rates have low surface-SOD immunofluorescence and low total SOD activity. Surface-SOD immunofluorescence can be measured, for example, by flow cytometry using sheep anti-Cu/Zn-SOD IgG polyclonal antibodies and FITC-

conjugated rabbit anti-sheep secondary antibodies (Alvarez, J.G. 18th Annual Meeting American Andrology Society, Tampa, FL., 1993, abstract 170). Greater than about 300 FITC units is indicative of high pregnancy potential, while less than about 300 FITC units is indicative of low pregnancy potential.

Ratio of unsaturated fatty acids to saturated fatty acids Unsaturated fatty acids are prone to oxidation while saturated fatty acids are insensitive to this process. Determination of the ratio of the unsaturated fatty acid, docosahexaenoic acid (22:6) to the saturated fatty acid palmitic acid (16:0) or of other unsaturated fatty acids to saturated fatty acids can be used to predict pregnancy potential. Samples that oxidize at high rates will have low docosahexaenoic acid/palmitic acid ratios. Unsaturated/saturated fatty acids can be measured by gas chromatography following alkaline methanolysis with 1N sodium methoxide at 40C for 1 hour (Alvarez, J.G. and J.C. Touchstone, Practical Manual on Lipid Analysis. Series of Monographs: I- Fatty Acids. Norell Press (New Jersey), 1991). A ratio of unsaturated to saturated fatty acids of greater than or equal to about 1.0 indicates high pregnancy potential; a ratio greater than or equal to about 0.5 and less than about 1.0 indicates borderline pregnancy potential; and a ratio of less than about 0.5 indicates low pregnancy potential.

Creatine kinase activity The concentration of creatine kinase in sperm reflects the degree of cytoplasmic extrusion during the last phase of spermatogenesis. Samples exhibiting abnormally high levels of creatine kinase activity have also been found to have high rates of lipid peroxidation as determined by the PE/PC ratios (PE/PC ratios < 0.5 and increased malonaldehyde production). This correlation supports the detection of creatine kinase activity as a means for determining pregnancy potential of a sperm sample. Creatine kinase activity in human sperm can be measured by spectrophotometric analysis at 365 nm of the NADPH generated during reaction of creatine kinase with ADP to produce creatine and ATP, followed by reaction of hexokinase with ATP and glucose to produce glucose-6-phosphate, followed by reaction of glucose-6-phosphate dehydrogenase with glucose-6-phosphate and NADP to produce NADPH. The NADPH generated under these conditions is proportional to the activity of creatine kinase in human sperm (Huszar, G. (1988) *Gamete Res 19*:67.). Creatine kinase (CK) exists in two isoforms: MM and BB. Sperm with high pregnancy potential have a CK-MM/CK-MM+CK-BB ratio of greater than or equal to about 10%.

Table 2
Pregnancy Potential or Contraceptive Efficacy Values from Direct Indicators of Lipid Peroxidation

Indicator	High Pregnancy Potential	Low Pregnancy Potential
SOD Activity	≥ 9 U/10^8	<7U/10^8
oxo^{8}dG	<20 fmol/ µg DNA	>40 fmol/ µg DNA
Surface SOD Immunofluorescence	>300 FITC	<300 FITC
Unsaturated/Saturated Fatty Acid Content	≥ 1.0	< 0.5
CK-MM/CK-MM + CK-BB	$\geq$ 10%	< 10%

Lipid peroxidation indicator values or changes in value in response to a stress can be quantitated by techniques and devices, which are well-known to one of skill in the art. The particular lipid peroxidation indicator chosen may be dictated by the degree of accuracy required and the availability of instruments to detect the results.

In a preferred embodiment for use as a kit, which is described in detail in the following Example 4, the indicator of sperm lipid peroxidation is based on sperm superoxide dismutase (SOD) activity. In a particularly preferred embodiment, sperm SOD activity is tested using an SOD antibody. As used herein, an antibody for use in detecting a sperm lipid peroxidation indicator can be any material that binds an antigen (e.g. polyclonal, monoclonal or single chain antibody or antibody fragment, such as an Fab of Fab'$_2$ fragment).

Immunodetection of sperm SOD or another antigenic indicator of lipid peroxidation can be accomplished using any of a number of competitive or non-competitive assay procedures. In general competitive immunoassays are performed by adding SOD to a sperm containing sample, so that the sperm and the SOD compete for a limited number of antibody binding sites resulting in the formation of sperm-antibody and labeled SOD-antibody complexes. By maintaining the concentration of labeled SOD and SOD antibody constant, the amount of labeled SOD-antibody complex formed is inversely proportional to the amount of sperm present in the sample. A quantitive determination of the sperm SOD can therefore be made based on the labeled SOD-antibody complex. Competitive assays can be homogeneous (i.e. not requiring separation of antibody bound tracer (e.g. labeled SOD) from free tracer, since the antigen-antibody interaction causes, directly or indirectly, a measurable change in the signal obtained from the label group of the tracer). Alternatively, competitive assays can be heterogeneous (i.e. requiring separation of bound tracer from free tracer prior to determining the amount of ligand in the sample).

In contrast to competitive immunoassays, non-competitive assays involve incubating a sperm containing sample with an immobilized SOD antibody for a period of

time sufficient to reach equilibrium with regard to the formation of antibody-sperm conjugates. The SOD antibody can be directly or indirectly labeled. For example, indirect labeling can be carried out after a wash step to remove unbound sperm by contacting the immobilized antibody-sperm complexes with a second, labeled antibody that is specific for the antibody-sperm complex. Following a second wash step to remove unbound second antibody, the amount of bound second antibody can be detected and measured as an indication of bound sperm. An example of this procedure is described in the following Example 5.

Exemplary competitive and non-competitive immunoassays include fluorescence polarization immunoassay (FPIA), fluorescence immunoassay (FIA), enzyme immunoassay (EIA), nephelometric inhibition immunoassay (NIA), enzyme linked immunosorbent assay (ELISA) and radioimmunoassay (RIA). General techniques for performing the various immunoassays are known to one of skill in the art. Moreover, a general description of most procedures is provided in U.S. Patent No. 5,051,361, which is incorporated herein by reference. The antibodies can be labeled in any manner that facilitates detection. Preferred labels include enzymes (e.g. horseradish peroxidase, alkaline phosphatase, urease, β-galactosidase), enzyme co-factors, radioisotopes (e.g. ^{3}H, ^{14}C, ^{125}I, ^{32}P, ^{131}I and ^{35}S), fluorescent compounds (e.g. fluorescein, rhodamine, allophycocyanin, phycoerythin, erythrosin, europian, luminol, luciferin and coumarin) and colored or uncolored beads or particles (e.g. silica gel, controlled pore glass, magnetic, Sephadex/Sepharose, cellulose, metal (e.g. gold) or latex). Preferred supports for immobilizing antibodies include membranes (e.g. polyethylene, polypropylene, polyamide, polyvinylidenedifluoride, glass fiber, paper), beads or particles and tubes, (e.g., glass, plastic or metal capillaries, straws or pipettes).

Based on the above-described procedures for identifying sperm samples with high pregnancy potential, the invention also features processes for increasing the success rate for initiating a preganancy using an assisted reproductive technology (ART) (i.e. a procedure for contacting a sperm with an ovum to initiate a pregnancy). Examples of ARTs include in vitro fertilization (IVF), gamete intrafallopian transfer (GIFT) intrauterine insemination (IUI) and intracytoplasmic sperm injection (ICSI). Procedures for performing ARTs are well-known to practitioners. It is expected that additional ARTs will be developed over time.

A preferred process of the invention involves obtaining multiple sperm samples from a donor (e.g. a male partner from a couple undergoing an ART) over time. For example, a donor can provide a new sperm sample once every other day. An aliquot (e.g. 1x 10^6 cells) of each sample can then be obtained for testing, while the remainder can be banked (e.g. cryopreserved) for potential future use in an ART. Preferably the process includes a

means for correlating a particular aliquot with the banked sample from which it was obtained. For example, an aliquot taken from a sample, as well as the sample itself obtained on day 1 can be labelled #1. A subsequently obtained sample from the same donor and an aliquot taken from that sample can be labelled #2, etc..

All aliquots provided by a particular donor can then be tested to identify sperm samples having "high pregnancy potential" (e.g. sperm having a greater than 50% probability of initiating a pregnancy upon contacting an oocyte). The sperm sample indicated as having the higher pregnancy potential can then be used in an ART and the remaining, lower pregnancy potential samples can be discarded.

The following Example 2 provides the results of a blind prospective cohort study of 33 couples undergoing in vitro fertilization (IVF) and 11 couples undergoing gamete intrafallopian transfer (GIFT), no successful pregnancies resulted from sperm samples which exhibited a stress test score of less than about 0.8. In contrast, when sperm samples having a stress test score of about 0.8 or greater were used in an ART, a pregnancy resulted 55% of the time. Therefore, where the indicator of lipid peroxidation is loss of motility, a stress test score of less than about 0.8 indicates low pregnancy potential, while a stress test score of about 0.8 or above indicates high pregnancy potential and is suitable for use in an ART.

Table 3 shows how the stress test score and therefore the pregnancy potential of sperm obtained from the same male can vary with time.

Table 3

ST scores for IVF male partners

Patient	Date of IVF	ST score
1.	11/05/93	0.35
	04/20/94	0.88
2.	11/15/93	0.88
	09/27/94	0.88
3.	11/17/93	0.88
	06/28/94	0.88
4.	01/24/94	1.00
	02/16/94	0.50
5.	01/05/94	0.20
	06/20/94	0.92
6.	05/03/94	1.00
	06/29/94	0.40
7.	05/05/94	1.00
	06/27/94	0.50
8.	02/22/94	0.80
	10/05/94	1.00
9.	06/28/94	1.00
	10/18/94	1.00

In addition, Figure 4 shows that sperm samples with stress test scores greater than about 0.8 have a high motility recovery, even after cryopreservation. Therefore it appears that a further benefit of using sperm samples indicated as having high pregnancy potential in an ART results from the fact that such samples are not damaged by cryopreservation to the same degree as sperm with low pregnancy potential.

In contrast to high pregnancy potential sperm samples, identification of sperm samples with low pregnancy potential can also be useful, for example, as a screen for detecting potentially infertile males. In addition, since even fertile males can occassionally generate ejaculates with low pregnancy potential, by identifying a sperm sample as having low pregnancy potential, use of the sample in an ART can be avoided. Further, by identifying sperm samples with low pregnancy potential, the user can determine whether a particular male contraceptive is effective or has taken effect. For example, a vasectomy typically requires a phase in period in which to take effect. The use of assays to identify

sperm samples with low pregnancy potential, as disclosed herein, is therefore useful for confirming the efficacy of a male contraceptive.

Low pregnancy potential sperm samples can be identified by lipid peroxidation tests as described above. In addition, low pregnancy potential sperm samples can be identified based on quantification of spermatozoa (non-motile and motile) in a sperm containing sample (e.g. ejaculate). If the number obtained is less than about 20 million spermatozoa/mL, the sperm sample is considered to have low pregnancy potential.

Examples of sperm targets or sperm components that can be quantitated as an indication of the number of sperm present in a sperm containing sample, include a sperm protein (e.g. a sperm flagella protein, a glycolytic enzyme, an antioxidant enzyme (e.g. glutathione peroxidase or superoxide dismutase), a nuclear protein, an acrosomal protein, α-tubulin, lactate dehydrogenase (LDH-X), protamine, acrosin or a mitochondrial protein.); or a sperm lipid (e.g. cholesterol, a phospholipid, a glycolipid, a triglyceride, a fatty acid, phosphatidylglycerol, seminolipid, and a docosahexaenoic acid). Preferred targets for sperm quantitation are selective for and abundant on sperm cells.

Preferably a sperm reagent (e.g. sperm antibody, ligand, lectin or substrate) is used to quantitate sperm. Preferred sperm reagents include labeled (e.g. enzyme, tracer (e.g. radioactive), dye or color particle labeled) or unlabeled anti-sperm antibodies (e.g. anti-human sperm polyclonal antibody; Arnel Products Co., Inc. Cherokee Station, New York, N.Y.; Chemicon International Inc., Temecula, California) or labeled or unlabeled antibodies against a sperm component (e.g. a sperm protein or sperm lipid). Further preferred sperm reagents include labeled (e.g. dye or tracer labeled) or unlabeled reagents that interact with a sperm protein (e.g. Protein Reagent (0.3% tetrabromophenol, Miles Scientific, Connecticut) or rhodamine 123, which accumulates within sperm mitochondria. Alternatively, the labeled reagent (e.g. rhodamine) can interact with a sperm lipid.

Preferred methods for counting sperm employ detectably labeled antibodies that specifically bind a sperm antigen (e.g. anti-human sperm polyclonal antibodies and antibodies specific to an epitope of the sperm flagellum, nuclear proteins, glycolytic enzymes, acrosome etc.). One method for quantitating sperm involves incubating a sperm sample with colored particles containing anti-sperm antibodies for an appropriate period of time to allow the sperm antigens to react with the antibody bound colored particles; ii) filtering the sample of step i), so that sperm/colored particle/antibody complex is retained on the filter and unbound, colored particle/antibody and seminal plasma protein passes through the filter; and iii) visualizing the color intensity.

For example, if colored particles are used, sperm/colored particle/antibody complexes can be quantitated by comparing the color of the filter to a color chart, which depicts various color possibilities for various quantities of sperm. Less than about 20 million spermatozoa/mL indicates low pregnancy potential. Alternatively, the filter membrane can be configured to indicate a "+" (i.e. fertility), if a sufficient amount of sperm/detectable particle/antibody complex is retained on the filter membrane to indicate that the sample is suitable for initiating a pregnancy (greater than about 20 million spermatozoa/mL) and a "-" (i.e. infertility) if an insufficient amount of sperm/detectable particle/antibody complex is retained on the filter membrane (less than about 20 million spermatozoa/mL).

In order to use antibodies or reagents that may also react with seminal plasma components present in a sperm containing sample (e.g. ejaculate), sperm can first be isolated. Seminal plasma-free sperm can then be contacted with anti-sperm antibody coated colored particles. After a sufficient period of time to allow antibodies and antigens to react, unbound antibody coated colored particles can be removed from the mixture and sperm/colored particle/antibody complex detected and quantitated. For example, the quantity of sperm in the sample can be quantitated using a color chart or a "+" or "-" filter as described above. Alternatively, sperm can be quantitated by detecting the appearance of agglutination in a drop of sample following addition of anti-sperm antibodies with bound latex particles.

Alternatively, low pregnancy potential can be determined by quantitating only motile sperm in a sperm sample. Motile sperm can be isolated from a sperm containing sample, for example, using the devices and procedures described above. Preferred methods for quantitating sperm employ detectably labeled antibodies that specifically bind a sperm antigen. Motile sperm can be quantitated using detectably labeled, sperm specific antibodies, such as anti-glutathione peroxidase (GPx) antibody. As with sperm SOD, immunodetection of sperm GPx or other sperm antigen can be accomplished using any of a number of competitive or noncompetitive assay procedures. In addition, sperm specific antibodies can be labeled (e.g. enzymatically) and if necessary immobilized by procedures that are well known in the art or as described above for SOD antibodies.

In a further aspect, the invention features sperm diagnostic kits or systems comprising a number of simple reagents and devices packaged in a box. The kit components can include one or more of the devices or components illustrated in Figures 1-3, and reagents for determining the pregnancy potential of sperm samples. Preferable kits include reagents for liquefying semen (e.g. chymotrypsin or pronase). For identifying sperm samples with high pregnancy potential (i.e. Male Fertility kit), preferable kits are based on measuring an indicator of lipid peroxidation or a change in an indicator over a period of time under defined conditions. Alternatively, as further described in the following Examples, a kit can be based

on determining the number of spermatozoa present in a sperm sample, wherein greater than about 40 million motile spermatozoa/mL indicates high pregnancy potential; less than about 20 million motile spermatozoa/mL indicates low pregnancy potential; and greater than about 20 million, but less than about 40 million indicates borderline pregnancy potential.

Preferable kits for identifying a sperm sample with low pregnancy potential, (e.g. to confirm the effectiveness of male contraception) are based on determining the number of spermatozoa/mL present in the sample, wherein less than about 50,000 spermatozoa/mL indicates low pregnancy potential or successful contraception. Reagents and devices for use in determining sperm pregnancy potential can include dispensing devices (e.g. capillaries or pipettes) for delivering a defined volume of isolated sperm from a collection container into a container which includes a means for determining the pregnancy potential of the sample, for example by quantitating the extent of sperm lipid peroxidation or change in lipid peroxidation resulting from a stress; and/ or sperm count.

In a preferred embodiment, the means for detecting sperm lipid peroxidation is based on sperm superoxide dismutase (SOD) activity and/or the means for detecting sperm count is based on sperm protein concentration. In a particularly preferred embodiment, the means for detecting sperm SOD activity employs a colorimetrically labeled SOD antibody and the means for detecting sperm protein employs gold particles. The kit also preferably includes a color chart against which concentrations of SOD and/or protein can be compared against known values. The kit can be optimized to develop color based on the protein levels corresponding to sperm concentrations greater than or equal to about 50,000 spermatozoa/mL.

A preferred kit for home determination of the pregnancy potential of a sperm sample can include: i) a collection container (optionally conformed to capture ejaculate, and/or containing a solution (e.g. enzymatic) that facilitates semen liquefaction); ii) a second container in which is contained anti-sperm antibodies covalently bound to colored particles in a hypotonic solution; iii) a membrane or filter for capturing sperm/colored particle or sperm/colored particle/antibody complexes and allowing passage of unbound, colored particle or colored particle/antibody; and iv) a chart for interpreting the results obtained based on the color of the filter. Indication of less than about 20 million spermatozoa/mL indicates low pregnancy potential when using the Male Fertility approach; or less than about 50,000 spermatozoa/mL when using the Male Contraceptive approach.

The present invention is further illustrated by the following Examples which are intended merely to further illustrate and should not be construed as limiting. The entire contents of all of the references (including literature references, issued patents, published

patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

Example 1: Kit for Isolating Motile Sperm From a Semen Sample

Kit Components:

1) (1) container (tube #1) for collecting and liquefying ejaculate containing 30mg/mL of dextran and 5mg/mL of chymotrypsin in an isotonic solution;

2) (1) see-through container (tube #2), which indicates three volumes: lower (1 mL), middle (1.5 mL) and upper (2 mL). The three volumes can be indicated by black and red marks (the top and the bottom mark being of the same color);

3) 0.5 mL container of isotonic solution (tube #3)

3) (1) 0.5 mL container of bovine serum albumin (BSA) at 0.2 mg/mL in an isotonic solution (tube #4);

4) (1) empty container;

5) (at least 3) disposable sterile pipettes or capillaries;

6) (1) capillary pipette to remove and transfer motile sperm

7) (at least 1) test strip containing a means for quantitating sperm (e.g. protein reagent);

8) instructions for use

9) (1) heating block at 37°C (optional)

Procedure:

The ejaculate was collected in the collection tube (tube #1) containing 5mg/mL of chymotrypsin and 30mg/mL of dextran in an isotonic solution. The mixture was allowed to stand at room temperature for about 5 minutes.

Using a dropper, 1 mL aliquots of the semen/dextran/chymotrypsin mixture were added to the calibrated see-through container (tube #2), so that the mixture reached the first marked level. The pipette was disposed of.

Using a second dropper, an appropriate volume of the isotonic solution (container #3) was used to overlay the semen/dextran/chymotrypsin mixture to the upper mark of each see-through container, resulting in the formation of a two layer density gradient: the lower layer consisting of the semen/dextran/chymotrypsin mixture and the upper layer consisting of the isotonic solution. The dropper was disposed of.

The mixture was allowed to stand for at least 5 minutes at room temperature or optionally in a 37°C heating block.

A sterile capillary pipette was inserted to reach the middle mark of the container and applied to a test strip which contained a means for quantitating sperm, e.g., protein reagent, lipid reagent.

Example 2: Temperature Stress for Predicting Pregnancy Potential of a Sperm Sample

Human sperm (HS) samples were obtained from 44 male partners of couples undergoing in vitro fertilization (IVF) (n=33) or gamete intrafallopian transfer (GIFT) (n=11). All male partners had normal semen by conventional analysis (By convention, a normal semen sample is defined as having greater than 20 million cells/mL, greater than 60% motility, a rate of progression greater than 2 (on a scale of 1 to 4), greater than 60% normal forms, 1.5 to 5.0 mL of ejaculate, no significant sperm agglutination, no significant number of leucocytes, and no hyperviscosity). Sperm samples were washed using the standard Percoll procedure and resuspended in HTF medium containing 10% plasmanate. An aliquot of the sperm suspension was utilized for artificial reproductive technologies (ART, e.g. IVF and GIFT) and a 100µL aliquot of the same sperm suspension was incubated at 40°C for 4h (stress test). Stress test scores were expressed as the ratio of final to initial motility. Analysis was performed by stepwise multiple regression. The independent variables were: women's age; HS motility before and after the stress test; stress test score; and the number of embryos transferred per IVF cycle. The dependent variables were: pregnancy and fertilization outcome.

In a blind cohort study, of the 44 ART cycles, 11 (25%) resulted in pregnancy. Of the 44 sperm samples used in the study, 24 (55%) had stress test scores $\leq$ 0.8 and 20 (45%) > 0.8. Controlling for all variables, the stress test score was significantly correlated with pregnancy outcome ($P = 0.0001$). In sharp contrast, the motility before the stress test was not correlated with pregnancy outcome. All pregnancies occurred in sperm samples with stress test scores > 0.8. Therefore, a cut-off value > 0.8 was selected to predict pregnancy outcome. The sensitivity of the stress test was 100% and the specificity was 73%. The negative predictive value of the test, defined as the absence of pregnancy with stress test scores < 0.8, was 100% and the positive predictive value, defined as the occurrence of pregnancy with stress test scores > 0.8, was 55%. Although the stress test score is highly predictive of pregnancy outcome, the fertilization rate was better predicted by the motility after the stress test ($P = 0.007$).

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Example 3: Oxidative Stress for Predicting Pregnancy Potential of a Sperm Sample

A similar test as described in Example 4 was carried out, except that the stress was provided by adding 10µL of 0.125mM ferrous iron in PBS (phosphate based saline) and 0.6mM ascorbate also in PBS to 30µL sperm suspensions for a total volume of 50 µL and incubating the mixture for 0.5h at 37°C with gentle shaking. Results indicate that the values obtained correlate with the stress test score at 40°C for 4h.

Example 4: Superoxide Dismutase (SOD) Activity in Sperm Correlates with Pregnancy Rate

Human spermatozoa release oxygen radicals and undergo spontaneous lipid peroxidation resulting in extensive damage to the sperm plasma and acrosomal membranes and loss of their pregnancy potential. Cu/Zn-superoxide dismutase (SOD) protects sperm against oxygen radical-mediated toxicity and endogenous lipid peroxidation. Surface SOD immunoreactivity is expressed over the mid piece and post equatorial regions of human sperm (Alvarez and Storey, (1992) *J. Andrology* 13(3):232-241, the contents of which are incorporated herein by reference).

Whether SOD activity correlates with sperm pregnancy potential and pregnancy rate was studied using sperm samples obtained from 27 male partners from couples undergoing in vitro fertilization (IVF). The age of the female partner ranged from 24 to 49 years. The number of oocytes inseminated ranged from 3 to 20 and the number of embryos transferred from 1 to 7. Sperm samples were washed using the standard mini Percoll procedure, and resuspended in HTF medium containing 10% plasmanate. An aliquot of the sperm suspension was utilized for IVF and another aliquot was used to measure the SOD activity of that particular sample.

SOD activity in the sperm samples examined ranged from 1.5 to 12 U/10^8 cells ($P < 0.001$). All six sperm samples with SOD activities less than or equal to 3.3U/10^8 cells resulted in failed fertilization ($P < 0.001$). Between 4.7 and 7.1U/10^8 cells, fifteen out of sixteen oocytes were fertilized but no ongoing pregnancies resulted.

These results show that sperm samples with high SOD activity (i.e. greater than about 9 units/ 10^8 cells) have a high pregnancy potential, while samples with low SOD activity (i.e. less than about 7 units/ 10^8 cells) have a low pregnancy potential. Samples having an SOD activity in the range of greater than or equal to about 7 and less than about 9 units/10^8 cells have borderline pregnancy potential.

Example 5: Assay for Identifying a High Pregnancy Potential Sperm Sample

The ejaculate was collected in the collection tube (tube #1) containing 5mg/mL of chymotrypsin. After about three to five minutes (at which time the semen should be liquefied), an aliquot (about 50µL) of the liquefied semen was added to another test tube (tube #2), which contained horseradish peroxidase-conjugated sheep anti-human superoxide dismutase (SOD) IgG polyclonal antibodies (The Binding Site, Inc., 5889 Oberlin Drive, San Diego, CA 92121) and horseradish peroxidase-conjugated sheep anti-human glutathione peroxidase (GPx) IgG polyclonal antibodies (The Binding Site, Inc., 5889 Oberlin Drive, San Diego, CA 92121) dissolved in a hypotonic solution. After five minutes, a dip test strip with bound sheep anti-human SOD and anti-human GPx Ig G polyclonal antibodies (The Binding Site, Inc., 5889 Oberlin Drive, San Diego, CA 92121) was inserted into test tube#2. After five minutes, the test strip was removed from the tube and rinsed with tap water.

The test strip was then dipped into another test tube (tube #3), which contained the peroxidase substrate, hydrogen peroxide (H_2O_2) and diaminobenzidine (DAB); (Sigma Chemical Co., St. Louis, MO 63178). The color ratio of SOD/GPx provides an indication of pregnancy potential by comparison with a chart provided with the kit. Color in the GPx pad indicates equal or greater than about 20 million spermatozoa/mL. If the color in the SOD pad is higher than the color in the GPx pad, a high pregnancy potential of the sperm is indicated. If the color in the SOD pad is equal to or lower than the color in the GPx pad, a low pregnancy potential is indicated.

Example 6: Assay for Monitoring the Efficacy of Male Contraception (Color Chart Approach)

The ejaculate from a male who had taken an appropriate dose of a male contraceptive that suppresses spermatogenesis (e.g. a GnRH antagonist) or who had had a vasectomy, was collected in the collection tube (tube #1) containing 5mg/mL of chymotrypsin in an isotonic solution. After three to five minutes (at which time the semen should be liquefied) about 500 µL of the liquefied semen was added to another test tube (tube #2) containing anti-human sperm polyclonal antibodies (Arnel Products Co., Inc., Cherokee Station, New York, N.Y.; Chemicon International Inc., Temecular, CA) covalently bound to gold particles (having an intrinsic pink color) or to colored latex particles (having a blue or yellow color) in a hypotonic or isotonic solution. The mixture was incubated for up to about 10 minutes to allow the sperm antigens exposed after the hypotonic treatment to react with the antibodies. Following incubation, the contents of test tube #2 was transferred to a filter membrane, so that sperm/colored particle/antibody complex was retained and unbound.

colored particle/antibody passed through the filter into a reservoir. The color of the filter can then compared to various color possibilities depicted on a chart that is included with the kit to determine the number of sperm present in the sample and thereby determine the efficacy of the male contraceptive treatment. This approach allows one to detect in semen a concentration of at least 50,000 spermatozoa/mL. This sensitivity is comparable to that obtained with the regular light microscope commonly used in andrology laboratories.

Example 7: Assay for Monitoring the Efficacy of Male Contraception (Plus (+) or Minus (-) Approach)

The ejaculate was collected in the collection tube (tube #1) containing 5mg/mL of chymotrypsin in an isotonic solution. After about 3 to 5 minutes (at which time the semen should be liquefied) a drop (50µl) (Male Fertility Kit) or 0.5 mL (Male Contraceptive Kit) of the liquefied semen was added to another test tube (tube #2) containing a hypotonic solution. After a period of incubation of 5 minutes, a drop (about 50µL) (Male Fertility Kit) or the total contents (Male Contraceptive Kit) was transferred to a membrane provided with two windows: window #1 was used to apply the sample where after the sample was applied, the soluble sperm antigens reacted with anti-human sperm antibodies conjugated to gold or colored latex particles included in a reservoir in the membrane; window #2 had a horizontal colored line. As the sperm antigen/antibody/particle complex migrated through the membrane, a second anti-human sperm antibody bound in the vertical position to the membrane underlying window #2 (capture antibody) reacted with the sperm antigen/antibody/particle complex producing a colored vertical line when the sperm concentration in semen was above 50,000 spermatozoa/mL in which case a plus (+) sign appeared. If the concentration of sperm in semen was below 50,000 spermatozoa/mL, a minus (-) sign appeared.

Example 8: Male Fertility & Contraceptive Screening Kits (Filter Approach)

Kit Components

1) (1) calibrated semen collection tube;
2) (1) squeeze bottle containing the chymotrypsin solution;
3) (1) test tube containing the isotonic or hypotonic solution;
4) (2) droppers;
5) (1) filter mounted on a liquid reservoir;
6) (1) squeeze bottle containing anti-sperm antibody-coated colored latex or gold particle solution;
7) instructions for use.

Procedure

Using the squeeze bottle, chymotrypsin solution (dextran-free) was added to the semen collection tube in a volume equal to the semen volume. The mixture was allowed to stand at room temperature for about 5 minutes in order for the semen to liquefy.

Using a dropper, one drop (Male Fertility Kit) or 0.5 mL (Male Contraceptive Kit) of the semen/chymotrypsin mixture in the semen collection tube was added to another test tube (test tube #2) containing an isotonic solution (e.g. phosphate buffered saline or Earle's Medium) or a hypotonic solution (e.g. distilled water) .

One drop (50µL) (Male Fertility Kit) or 0.5 mL (Male Contraceptive Kit) of test tube #2 was added to a filter (e.g. blocked nitrocellulose, 5µm pore size or microfiber glass filter, 2.7 µm pore size) mounted on a liquid reservoir. Seminal plasma proteins, which were about 10-20 nm in size passed through the filter, while sperm cells which were about 1000 times bigger (50µm in size) were retained.

One drop of anti-sperm antibody-coated colored particles (e.g. gold particles that have an intrinsic pink color; or a colored latex particle) was added onto the filter and the appearance of color on the filter indicated a potentially fertile sperm sample. By using this filter procedure, the antibody used to coat the latex particle did not have to be highly specific for the sperm cell (i.e. it could exhibit some cross-reactivity with seminal plasma proteins).

Example 9: Male Fertility & Contraceptive Screening Kits (Filter Approach with Preincubation of the Antibody and the Sperm Antigens)

Kit Components

1) (1) calibrated semen collection tube;
2) (1) squeeze bottle containing the chymotrypsin solution;
3) (1) test tube containing the isotonic or hypotonic solution;
4) (2) droppers;
5) (1) filter mounted on a liquid reservoir;
6) (1) squeeze bottle containing anti-sperm antibody-coated colored latex or gold particle solution;
7) instructions for use.

Procedure

Using the squeeze bottle, chymotrypsin solution (dextran-free) was added to the semen collection tube in a volume equal to the semen volume. The mixture was allowed to stand at room temperature for about 5 minutes in order for the semen to liquefy.

Using a dropper, one drop (about 50µL) (Male Fertility Kit) or 0.5 mL (Male Contraceptive Kit) of the semen/chymotrypsin mixture in the semen collection tube was added to another test tube (test tube #2) containing anti-sperm antibody-coated colored particles (e.g. gold particles that have an intrinsic pink color; or a colored latex particle) in an isotonic solution (e.g. phosphate buffered saline or Earle's Medium) or hypotonic solution (e.g. distilled water).

One drop (50µL) (Male Fertility Kit) or 0.5 mL (Male Contraceptive Kit) of the contents of test tube #2 was added to a filter (e.g. nitrocellulose, 5µm pore size or microfiber glass filter, 2.7 µm pore size) mounted on a liquid reservoir. Seminal plasma proteins/color particle antibody/complex, which were about 10-20 nm in size passed through the filter, while sperm cells/colored particle antibody/complex which were about 1000 times bigger (50µm in size) were retained. By using this filter procedure, the antibody used did not have to be highly specific for the sperm cell (i.e. it may show some cross-reactivity with seminal plasma proteins).

Example 10: Male Fertility & Contraceptive Screening Kits (Agglutination Approach)

Kit Components

1) (1) calibrated semen collection tube;
2) (1) squeeze bottle containing the chymotrypsin solution;
3) (1) test tube containing an isotonic or hypotonic solution and sperm-specific antibody-coated particles (uncolored or colored);
4) (2) droppers;
5) (1) slide having a color, which contrasts with the anti-sperm antibody-coated particles.
6) instructions for use.

Procedure

Using the squeeze bottle, chymotrypsin solution (dextran-free) was added to the semen collection tube (tube #1) in a volume equal to the semen volume. The mixture was allowed to stand at room temperature for about 5 minutes in order for the semen to liquefy.

Using a dropper, one drop (50µL) (Male Fertility Kit) or 0.5 mL (Male Contraceptive Kit) of the semen/chymotrypsin mixture was added to another test tube (test tube #2) containing an isotonic solution (e.g. phosphate buffered saline or Earle's Medium) or hypotonic solution (e.g. distilled water) and sperm-specific antibody-coated particles (uncolored or colored).

One drop of test tube #2 was deposited on a slide, which was of a color that contrasted with the sperm specific antibody coated latex particles, so that agglutination, indicating the potential presence of fertile sperm could be discerned on the slide. The absence of agglutination when using the Male Fertility approach, was indicative of less than about 20 million spermatozoa/mL; borderline agglutination was indicative of greater than about 20 million spermatozoa/mL and less than about 40 million spermatozoa/mL; and the presence of high agglutination was indicative of greater than about 40 million spermatozoa/mL. The absence of agglutination when using the Male Contraceptive approach was indicative of less than about 50,000 spermatozoa/mL.

Example 11: Male Fertility & Contraceptive Screening Kits (Non Immunoreactive/Filter Approach)

Kit Components

1) (1) calibrated semen collection tube;
2) (1) squeeze bottle containing the chymotrypsin solution;
3) (1) test tube containing the isotonic or hypotonic solution;
4) (2) droppers;
5) (1) filter mounted on a liquid reservoir;
6) (1) squeeze bottle containing colored reagents that react non-specifically with sperm cell components, e.g., pink-colored gold particles (reacts with proteins) or red-colored rhodamine (reacts with lipids).
7) instructions for use.

Procedure

Using the squeeze bottle, chymotrypsin solution (dextran-free) was added to the semen collection tube (tube #1) in a volume equal to the semen volume. The mixture was allowed to stand at room temperature for about 5 minutes in order for the semen to liquefy.

Using a dropper, one drop (50µL) (Male Fertility Kit) or 0.5 mL (Male Contraceptive Kit) of the liquefied semen was added to another test tube (tube #2) containing gold particles (that have an intrinsic pink color) in a hypotonic or isotonic solution. One drop (Male Fertility Kit) or 0.5 mL (Male Contraceptive Kit) of the contents of tube #2 was added onto the filter (nitrocellulose with 5µm pore size or microfiber glass filter with a pore size of 2.7µm). Gold particles have an intrinsic pink color and high affinity for biomolecules (e.g. proteins or lipids) and therefore can bind to both spermatozoa and seminal plasma proteins. By using this filter procedure, the unbound gold particles and the gold particles bound to seminal plasma proteins went through the filter into the reservoir while sperm cells with bound gold particles were retained on the filter indicating a pink color. The presence of a pink color when using the Male Fertility approach, was indicative of the presence of greater than about 20 million spermatozoa/mL; and greater than about 50,000 spermatozoa/mL when the Male Contraceptive approach was used.

Example 12: Assay for Identifying a High Pregnancy Potential Sperm Sample

The ejaculate was collected in the collection tube (tube #1) containing 5mg/mL of chymotrypsin in an isotonic solution. After about 3 to 5 minutes (at which time the semen should have been liquefied) a drop (about 50µL) of the liquefied semen was added to a test tube (tube #2) containing anti-glutathione peroxidase (GPx) antibodies bound to yellow colored particles and anti-superoxide dismutase (SOD) antibodies bound to blue colored particles in a hypotonic or isotonic solution. The mixture was incubated for up to about 10 minutes to allow the sperm antigens exposed after the hypotonic treatment to react with the antibodies. Following incubation, the contents of test tube #2 was transferred to a filter membrane, so that sperm/colored particle/antibody complex was retained and unbound, colored particle/antibody passed through the filter into a reservoir. If the filter appeared green, the SOD/GPx ratio was about 1:1 and the sample had borderline pregnancy potential; if the filter appeared bluish, high SOD content was indicated and the sample had high pregnancy potential; and if the filter appeared yellowish, low SOD content was indicated and the sample had low pregnancy potential.

Example 13: Male Fertility & Contraceptive Screening Kit

Kit Components

1) (1) calibrated semen collection tube;
2) (1) squeeze bottle containing the liquefying enzyme (e.g. chymotrypsin or pronase at 5 mg/ml.) (squeeze bottle #1);
3) (1) squeeze bottle (with a removable top) containing 150µL of an isotonic or hypotonic solution (squeeze bottle #2);
4) (1) squeeze bottle containing a 0.5 mg/mL solution of Rhodamine-123 (Molecular Probes, Inc. Eugene, OR) in distilled water (squeeze bottle #3);
5) (1) dropper;
6) (1) porous filter mounted on a liquid reservoir;
7) instructions for use.

Procedure

Using the squeeze bottle provided (squeeze bottle #1), two drops (per milliliter of semen) of a solution of pronase solution (5mg/mL) (Sigma Chemical Corp., St. Louis, MO) in Earle's medium was added to the ejaculate in the collection tube. The mixture

was allowed to stand at room temperature for about 5 minutes in order for the semen to liquefy.

Using a dropper, one drop (Male Fertility Kit) or four drops (Male Contraceptive Kit) of the liquefied semen was added to another squeeze bottle (squeeze bottle #2) which had the top removed and contained 150µL (Male Fertility Kit) or 250µL (Male Contraceptive Kit) of a hypotonic solution. Then, one drop (Male Fertility Kit) or 4 drops (Male Contraceptive Kit) of the Rhodamine-123 (Molecular Probes, Inc. Eugene, OR) solution in squeeze bottle #3 was added to squeeze bottle #2 and the top placed back on. The contents were then mixed by tapping with a finger.

Two drops (Male Fertility Kit) or the total content of squeeze bottle #2 was added to a porous filter (about 2.7 µm pore size) mounted on a liquid reservoir and the color visualized. The absence of color was indicative of a concentration of less than about 20 million spermatozoa/mL; faint color was indicative of greater than about 20 million spermatozoa/mL and less than about 40 million spermatozoa/mL; and the presence of color was indicative of greater than about 40 million spermatozoa/mL.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A system for collecting motile sperm from a sperm containing sample, said system comprising:

a) housing means forming a housing having an internal cavity for holding a sperm containing sample; and

b) isolation means, selectively disposable within the cavity and in fluid communication with the sperm containing sample when disposed therein, for isolating motile sperm.

2. The system of claim 1 wherein said isolation means comprises a retainer being selectively and removably positionable within the cavity of the housing and being sized to accomodate the migration of the motile sperm from the sperm sample into an internal passageway formed therethrough, the motile sperm being isolated within the passageway after a sufficient period of time.

3. The system of claim 2 wherein said retainer comprises a fluid that is less dense than the sample.

4. The system of claim 1 wherein said retainer comprises a porous membrane.

5. A system of claim 1, which additionally comprises a means for testing the motile sperm to determine the pregnancy potential of the sample.

6. The system of claim 5 wherein said means for testing comprises a means for determining an indicator of lipid peroxidation or a change in an indicator of lipid peroxidation.

7. The system of claim 6 wherein said means for determining comprises a means for quantitating sperm superoxide dismutase activity using an appropriate superoxide dismutase antibody.

8. The system of claim 7 wherein said superoxide dismutase antibody is colorimetrically labeled.

9. The system of claim 5 wherein said means for testing comprises a means for determining the number of motile sperm within the sperm containing sample.

10. The system of claim 9 wherein said means for determining comprises a means for quantitating a sperm lipid or a sperm protein.

11. The system of claim 10 wherein the sperm protein is glutathione peroxidase.

12. An assay for determining the pregnancy potential of a sperm containing sample, comprising the steps of:

a) isolating sperm from a sperm containing sample, and

b) testing the sperm to determine the pregnancy potential of the sample.

13. An assay of claim 12 wherein the isolating step a) results in the isolation of mainly motile sperm from the sperm containing sample.

14. An assay of claim 13, wherein prior to the isolating step, an additional step of liquefying the sample is performed.

15. An assay of claim 12, wherein the testing comprises a means selected from the group consisting of quantitating an indicator of lipid peroxidation, quantitating a change in an indicator of lipid peroxidation and quantitating a sperm component sperm.

16. An assay of claim 15, wherein the indicator of lipid peroxidation is selected from the group consisting of a lipid peroxidation breakdown product, sperm phosphatidylethanolamine/phosphatidylcholine ratio and sperm motility.

17. An assay of claim 15, wherein the change in an indicator of lipid peroxidation is selected from the group consisting of: superoxide dismutase activity, oxo-8-deoxyguanosine level, surface superoxide dismutase immunofluorescence, ratio of saturated to unsaturated fatty acids and creatine kinase activity.

18. An assay of claim 15, wherein the sperm component is a sperm protein.

19. An assay of claim 18 wherein the sperm protein is selected from the group consisting of: a flagella protein, a glycolytic enzyme, glutathione peroxidase, a nuclear protein, a mitochondrial protein, an acrosomal protein, α-tubulin, lactate dehydrogenase (LDH-X), a sperm protamine and acrosin.

20. An assay of claim 15, wherein the sperm component is a sperm lipid.

21. An assay of claim 20, wherein the sperm lipid is selected from the group consisting of: phosphatidylglycerol, seminolipid, docosahexaenoic acid, cholesterol, a phospholipid, a glycolipid, a triglyceride, and a fatty acid.

22. An assay for determining the pregnancy potential of a sperm containing sample comprising the steps of:

a) contacting the sperm containing sample with an appropriate amount of a detectable sperm reagent, thereby forming a sperm reagent/sperm complex; and

b) quantitating the sperm reagent/sperm complex, to determine the pregnancy potential of the sperm containing sample.

23. An assay of claim 22, wherein prior to the isolating step, an additional step of liquefying the sample is performed.

24. An assay of claim 22, wherein the sperm reagent is an antibody against a sperm protein.

25. An assay of claim 24, wherein the sperm protein is selected from the group consisting of α-tubulin, lactate dehydrogenase (LDH-X), protamine, acrosin, a flagella protein, a glycolytic enzyme, glutathione peroxidase, a nuclear protein and a mitochondrial protein.

26. An assay of claim 22 wherein the sperm reagent is an antibody against a sperm lipid.

27. An assay of claim 26, wherein the lipid is selected from the group consisting of phosphatidylglycerol, seminolipid, docosahexaenoic acid, cholesterol, a phospholipid, a glycolipid, a triglyceride, and a fatty acid.

28. An assay of claim 22 wherein the sperm reagent is a dye or tracer that interacts with a sperm protein.

29. An assay of claim 22 wherein the sperm reagent is a dye or tracer that interacts with a sperm lipid.

30. An assay of claim 22, wherein the reagent interacts with a sperm antioxidant enzyme or a lipid peroxidation breakdown product.

31. An assay of claim 30, wherein the lipid peroxidation product is selected from the group consisting of oxo-8-deoxyguanosine level, ratio of saturated to unsaturated fatty acids and creatine kinase activity.

32. An assay of claim 22, wherein prior to step b), the sample of step a) is filtered, so that sperm reagent/sperm complex is retained on a filter and uncomplexed sperm reagent passes through the filter.

33. An assay of claim 32, wherein the sperm reagent is colored and the absence of color on the filter is indicative of less than about 20 million spermatozoa/mL and therefore of low pregnancy potential; the presence of a faint color is indicative of greater than about 20 and less than about 40 million spermatozoa/mL and therefore of borderline pregnancy potential; and intense color is indicative of greater than about 40 million spermatozoa/mL and therefore of high pregnancy potential.

34. An assay of claim 33, wherein the sperm reagent is selected from the group consisting of a protein dye and a lipid dye.

35. An assay of claim 22, wherein the sperm containing sample is a seminal plasma-free sperm sample.

36. An assay of claim 35, wherein the sperm containing sample consists only of motile sperm cells.

37. An assay of claim 22 wherein the sperm reagent is an anti-sperm antibody.

38. An assay of claim 37, wherein the anti-sperm antibody is labeled with a detectable particle.

39. An assay of claim 38, wherein the detectable particle is a gold particle or a colored latex particle.

40. An assay of claim 37 wherein the anti-sperm antibody is selected from the group consisting of an anti-human sperm polyclonal antibody and an anti-human glutathione peroxidase antibody.

41. An assay for identifying the pregnancy potential of a sperm containing sample, comprising the steps of:

a) contacting a sperm containing sample with a first detectable particle containing an anti-superoxide dismutase antibody and a second detectable particle containing an anti-glutathione peroxidase antibody thereby forming sperm/ first detectable particle complexes and sperm/ second detectable particle complexes;

b) filtering the sample of step a), so that sperm/ first detectable particle complexes and sperm/second detectable particle complexes are retained on a filter and unbound first detectable particles and second detectable particles pass through the filter; and

c) quantitating each of the first detectable particle and the second detectable particle, wherein a ratio of first detectable particle: second detectable particle below about 1 is indicative of a low pregnancy potential sample; a ratio above about 1 and below about 1.5 is indicative of a borderline pregnancy potential sample; and a ratio above about 1.5 is indicative of a high pregnancy potential sample.

42. An assay of claim 41 wherein the first detectable particle and second detectable particle are distinct and are selected from the group consisting of a gold particle and a colored latex particle.

43. An assay of claim 41 wherein step c), the ratio is determined by quantitating the color resulting from the mixture of the first detectable particle and the second detectable particle.

44. An assay of claim 41 wherein step c), the ratio is determined by comparing the presence of two different colors

45. An assay for determining the pregnancy potential of a sperm containing sample, comprising the steps of:

a) contacting a sperm containing sample with a detectable particle containing anti-sperm antibodies thereby forming sperm/detectable particle complexes;

b) depositing the sample of step a) onto a colored slide that contrasts with the color of the detectable particles; and

c) quantitating the sperm/detectable particle complexes wherein the absence of the detectable particle color is indicative of less than about 20 million spermatozoa/mL and therefore of a low pregnancy potential sample; faint detectable particle color is indicative of equal to or greater than about 20 and less than about 40 million spermatozoa/mL and therefore of a borderline pregnancy potential sample; and the presence of detectable particle color is indicative of greater than about 40 million spermatozoa/mL and therefore of a high pregnancy potential sample.

46. An assay of claim 45, wherein the detectable particle is a latex particle

47. An assay of claim 46 wherein the latex particle is colored.

48. A method for determining the pregnancy potential of a sperm containing sample, comprising the steps of:

a) obtaining multiple sperm containing samples from a donor over time;

b) obtaining an aliquot from each of the multiple sperm containing samples; and

c) testing each aliquot to determine pregnancy potential.

49. A method of claim 48, wherein step c), the pregnancy potential is determined based on determining an indicator of lipid peroxidation or a change in an indicator of lipid peroxidation.

50. A method of claim 49, wherein the indicator of lipid peroxidation is superoxide dismutase activity.

51. A method of claim 48, which additionally comprises the step of: d) using at least one sperm sample identified as having high pregnancy potential in an assisted reproductive technology.

52. A method of claim 51, wherein the assisted reproductive technology is selected from the group consisting of in vitro fertilization, gamete intrafallopian transfer, intrauterine insemination and intracytoplasmic sperm injection.

53. A system for quantitating the relative amounts of at least two analytes in a sample, said system comprising:

a) at least two reagents that specifically bind to each analyte,

b) means for producing a color upon interaction of the reagent with the corresponding analyte and

c) means for measuring the color produced upon mixing of the reagents.

54. A system of claim 53 wherein the reagents are antibodies with bound colored particles.

55. A system of claim 54 wherein the colored particles are latex particles.

56. A system of claim 55 wherein the latex particles have two different colors that produce a distinct third color.

57. A system of claim 56 wherein the preferred color pairs are blue-yellow and red-yellow, so that a 1:1 mixture produces green and orange, respectively.

58. A system of claim 53 wherein the analytes are sperm proteins.

59. A system of claim 58 wherein the sperm proteins are selected from a group consisting of superoxide dismutase and glutathione peroxidase.

60. A system of claim 57 wherein the analytes are sperm lipids.

61. A system of claim 60 wherein the sperm lipids are selected from a group consisting of phosphatidylglycerol, seminolipid, cholesterol and docosahexaenoic acid.

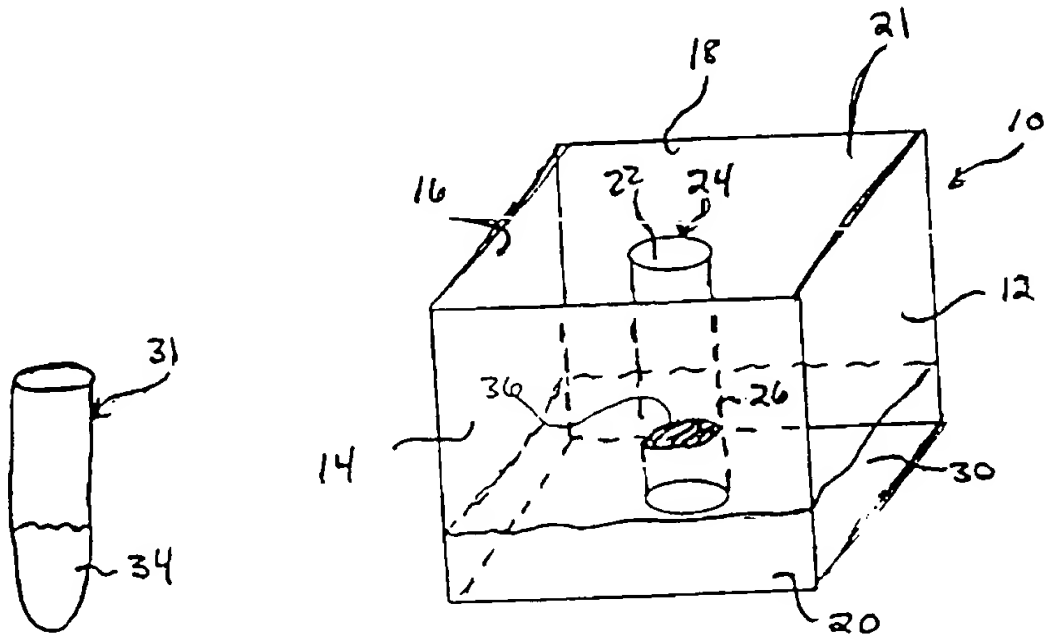


FIGURE 1

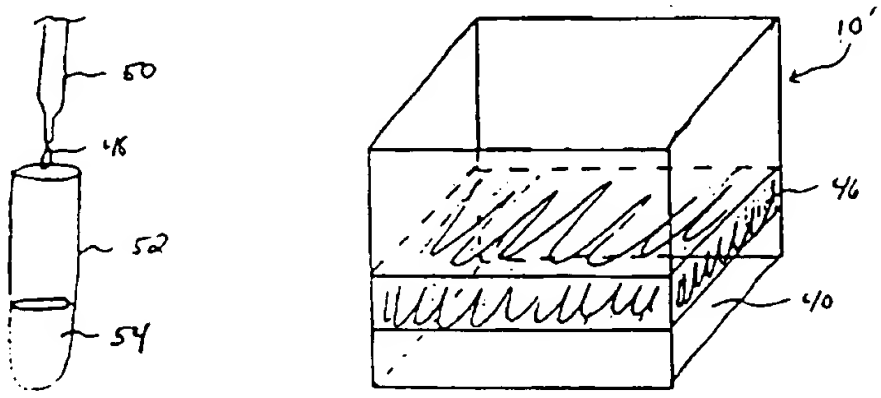


FIGURE 2

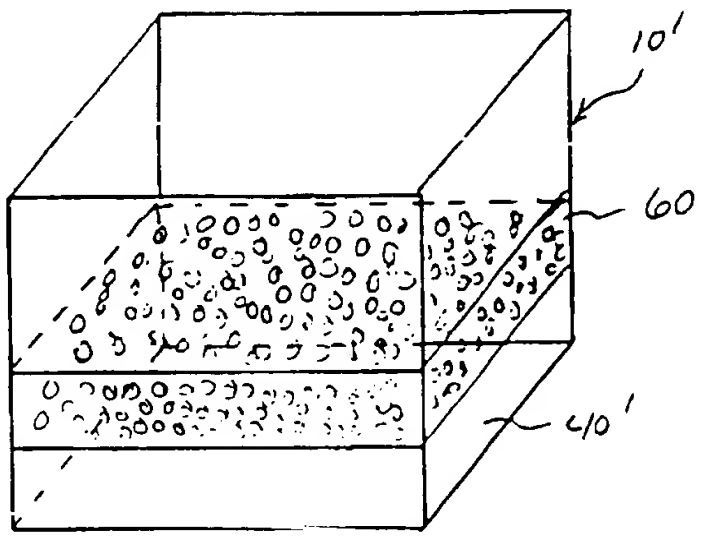
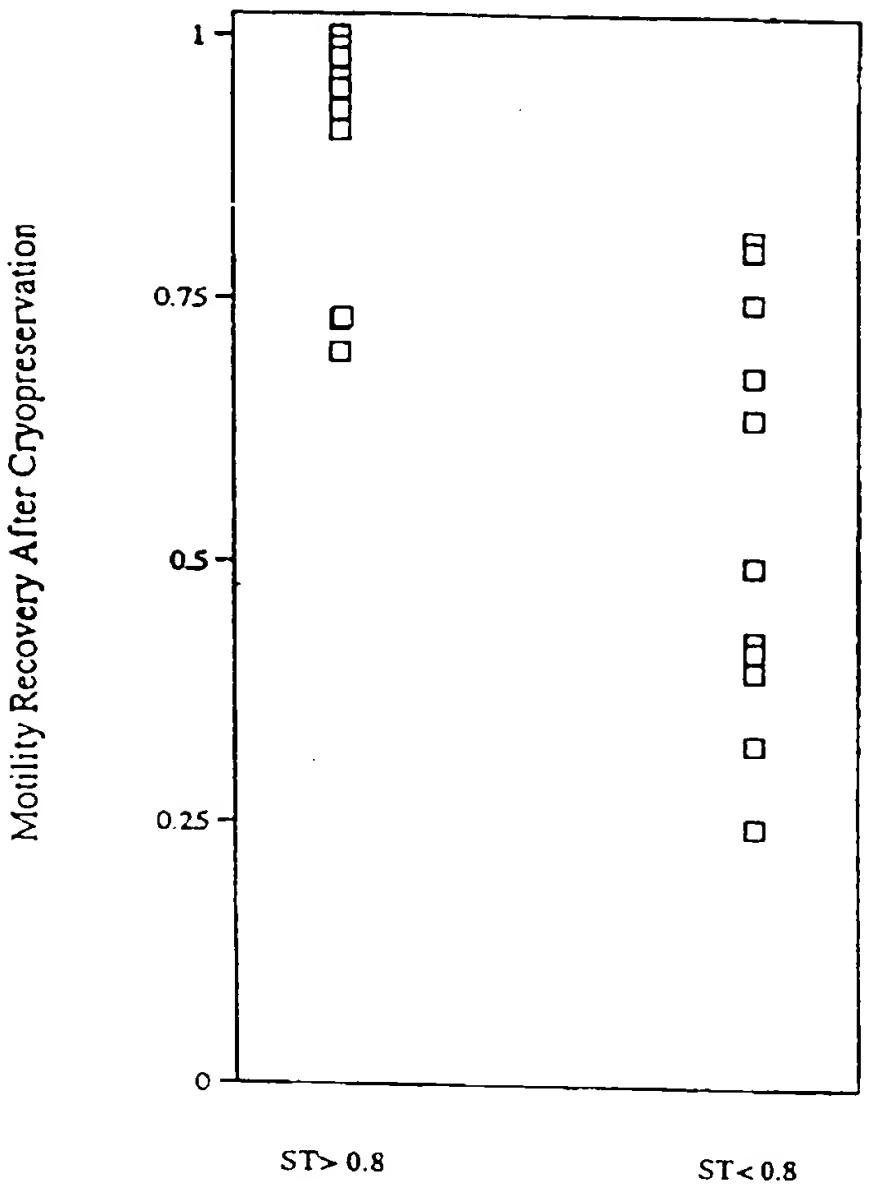


FIGURE 3

FIGURE 4


Motility Recovery After Cryopreservation
1
0.75
0.5
0.25
0
ST> 0.8
ST< 0.8

CORRECTED VERSION*

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(54) Title: ASSAYS, DEVICES AND KITS FOR DETERMINING MALE FERTILITY

(57) Abstract

Assays, devices and kits for identifying sperm samples with high pregnancy potential (e.g. for use in an assisted reproductive technology) or sperm samples with low pregnancy potential (e.g. for identifying potentially infertile males or for evaluating the effectiveness of a male contraception means) are disclosed.

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ASSAYS, DEVICES AND KITS FOR DETERMINING MALE FERTILITY

Background of the Invention

Determining the Fertility of a Sperm Sample

According to recent studies, male infertility is responsible almost 40% of the time that a couple is unable to conceive a child. In addition, use of male contraceptives is on the rise. For example, according to current estimates, more than 500,000 vasectomies are performed in the U.S. each year and about 2,000,000 are performed worldwide. In addition to vasectomies, a variety of oral contraceptives for use by males are in development. Although male contraceptives decrease the probability that a fertile male will initiate a pregnancy, no male contraceptive is 100% effective. Further, most male contraceptives require a certain period of time in which to take effect.

For a semen sample to be considered fertile according to standards set by the World Health Organization, at least two 1.5-5.0 milliliter ejaculate volumes obtained from a male must contain a sperm density of greater than 20 million spermatozoa/mL and/or a percent motility of 60% with a forward progression greater than 2 (on a 1-4 scale). In addition, the semen samples should show no evidence of sperm agglutination, pyospermia or hyperviscosity (Sigman, M., et al., Evaluation of the subfertile male. In: Lipschultz, LI and SS Howards eds. *Infertility in the Male*, 2nd ed. Chicago: Mosby-Year Book, 1991; p.184).

By convention, male infertility is diagnosed based on low sperm motility and/or count. However, motility analyses can produce false negatives, since viable sperm may appear non-motile due to damage sustained during processing. With regard to sperm count, 20 million spermatozoa/mL or greater is generally considered to be in the fertile range. However, non-motile, non-viable sperm can be included in the count. Sperm count and motility are typically assessed using commercially available instruments, such as light microscopes and computerized videoanalysis systems.

U.S. Patent No. 5,068,089 describes a home kit for testing fertility of human sperm based on ability of the sperm to reduce a dye. The extent of reduction (displayed colorimetrically), is said to be indicative of sperm fertilizing ability. However, this test is

time consuming, requires incubation at a temperature above room temperature and does not distinguish between reduction due to sperm cells or other cells, which may be present in a semen sample.

U.S. Patent No. 5,219,729 describes a laboratory assay for determining the fertilizing ability of sperm based on the affinity of binding to an oocyte zona pellucida fragment. The tighter the binding, the greater the fertilizing ability of the sperm sample. However, this assay requires freshly prepared oocyte fragments and at least four hour's time during which the sperm must be kept in contact with the oocyte fragment.

U.S. Patent No. 5,434,057 describes assays and kits for determing male fertility based on the detection of fumarase activity. However, since fumarase is ubiquitously present in cells including cells that may be present in a sperm sample (epithelial, leukocyte and bacterial or fungal), whether fumarase activity is an accurate indicator of sperm count and motility, as claimed in the patent, is dubious.

A simple, rapid and accurate assay for determining the fertility of a sperm sample in a reference laboratory, doctor's office or at home is needed.

Increasing the Effectiveness of an Assisted Reproductive Technology

Assisted reproductive technologies (ARTs), such as *in vitro* fertilization (IVF), gamete intrafallopian transfer (GIFT), intrauterine insemination (IUI) and sperm intracytoplasmic injection (ICSI) offer ways to initiate a pregnancy when natural approaches have been unsuccessful. These techniques are also useful for breeding animals or producing transgenic animals.

However, ARTs do not always result in a successful pregnancy. For example, IVF has an estimated success rate of about 25%, while GIFT is estimated as being successful in about 31% of attempts. One factor, which may contribute to an unsuccessful ART attempt is that not all sperm samples are capable of fertilization. According to recent studies, male infertility is responsible almost 40% of the time that a couple is unable to conceive a child.

In addition, the chance that a sperm sample will be incapable of initiating a pregnancy is increased when that sample has been stored for any period of time or cryopreserved. This finding has particular significance in view of the use of cryopreserved sperm in ARTs. Cryopreservation can result in sublethal cryodamage, in which cell viability post-thaw is lost more rapidly at later times than in fresh cells. Sublethal cryodamage has been shown to be due in part to membrane embrittlement during the phase transitions involved in freezing and thawing (Alvarez, J.G. and B.T. Storey (1993) *J. Androl.* 14 (3):

199-209). To a lesser degree, sublethal cryodamage ha· been shown to be caused by spontaneous lipid peroxidation (SLP) of sperm phospholipids (Alvarez, J.G. and B.T. Storey (1993) *J. Androl. 14* (3): 199-209 and J.G. Alvarez and B.T. Storey (1992) *J. Androl 13(3)*: 232-241). Spontaneous lipid peroxidation appears to be the major factor limiting the motile lifetime of sperm that has not been cryopreserved (Alvarez, J.G. and B.T. Storey (1988) *Gamet Res. 23*:77-90; and Alvarez, J.G. and B.T. Storey (1985) *Biol. Reprod. 32*: 342-351). The selective oxidation of phospholipid-bound polyunsaturated fatty acid moieties resulting from spontaneous lipid peroxidation leads to extensive oxidative damage to the sperm plasma and acrosomal membranes and DNA.

However, cryopreservation of sperm samples is routinely performed in ART procedures and in fact is required in order to test the donor for the presence of transmissible infectious agents (e.g., HIV) prior to insemination. For example, a donor is typically tested six months after producing a particular sample and only if the test is negative will the stored sample be used for insemination.

More and more couples are turning to ARTs to conceive a child. In addition, in vitro fertilization technologies are increasingly being used by breeders of livestock and in generating transgenic animals. A method for increasing the success rate of ARTs is needed.

Summary of the Invention

In general, the invention features devices, procedures and kits for isolating and quantitating motile sperm from a sperm-containing sample (e.g. semen) and/or for determining the pregnancy potential of a sperm sample. In general, the devices comprise a container for isolating a sperm containing sample, which is preferably adapted to separate sperm based on motility. The container can optionally be conformed to facilitate collection (e.g. funnel-shaped). In a preferred embodiment, a retainer for accomodating entry and migration of motile sperm, but not non-motile cells in the sperm containing sample, is positioned inside of the container. Preferably at an appropriate site within the retainer is positioned a means to determine the pregnancy potential of sperm that migrate into the retainer. Alternatively, isolated motile sperm can be obtained from the retainer and separately tested.

In one embodiment, the retainer is a compartment (e.g. a tube), which is in fluid communication with the sperm containing sample. In another embodiment, the retainer is a fluid, which is less dense than the sperm containing sample and thereby facilitates passage of motile sperm, but not non-motile cells. In a further embodiment, the retainer includes a porous membrane, which separates the sperm containing sample from an isolation area and which thereby prevents passage of non-motile sperm or other cells that may be

present in the sperm containing sample, but allows passage of motile sperm into the isolation area.

In a second aspect, the invention features assays for identifying sperm samples with high pregnancy potential. The assays can be used to provide an indication of the fertility status of the male donor or can be selected for use in an Artificial Reproductive Technology (ART). In a preferred embodiment, the process comprises the steps of: i) obtaining multiple sperm samples from a donor over time, ii) obtaining an aliquot from each sperm sample, iii) testing each aliquot to determine pregnancy potential, and iv) using at least one sperm sample having high pregnancy potential in an ART to initiate a pregnancy. In a preferred embodiment, the testing is based on quantitating an indicator of lipid peroxidation or a change in an indicator induced by a stress. A particularly preferred indicator of sperm lipid peroxidation is sperm superoxide dismutase (SOD) activity, which can be quantitated, for example, using an anti-SOD antibody.

In a third aspect, the invention features screening assays for detecting the pregnancy potential of sperm samples and thereby identifying potentially infertile males or evaluating males following vasectomy or once a particular contraceptive has been administered. In a preferred embodiment, the screening assay is based on determining motile sperm count. In one embodiment, motile sperm are first isolated and counted. Quantitation of less than about 50,000 motile spermatozoa/mL indicates that the contraceptive has been effective and that the sperm sample has low pregnancy potential. In another embodiment, quantitation of sperm is performed directly on the sperm containing sample (without separation of motile and immotile sperm) and quantitation of less than about 20 million spermatozoa/mL indicates that the sample has low pregnancy potential; greater than about 20 million spermatozoa/mL and less than about 40 million spermatozoa/mL indicates that the sample has borderline pregnancy potential; and greater than about 40 million spermatozoa/mL indicates that the sample has high pregnancy potential. The cutoff value of 20 million spermatozoa/mL includes both motile and immotile spermatozoa. Although this value is not diagnostic of infertility, it is intended to be used as part of a screening test. In this way, males that use the kit to test their fertility potential and have less than about 20 million spermatozoa/mL will be alerted to a potential infertility problem.

In a fourth aspect, the invention features sperm diagnostic kits or systems which comprise a number of simple reagents and devices packaged in a box. In one embodiment for use in identifying a high pregnancy potential sperm sample, the kit can include a sperm isolation means and a means for identifying a high pregnancy potential sperm sample. Optionally, the sperm isolation means is of an appropriate conformation (e.g. funnel shaped) to facilitate collection. Also optionally, a reagent for liquefying semen (e.g.

pronase or chymotrypsin is included in the kit for use in sperm isolation. Preferably the means for identifying sperm with high pregnancy potential is based on lipid peroxidation and the means for screening for pregnancy potential (e.g. infertility or effective male contraception) is based on sperm count.

The instant disclosed assays, devices and kits are easy to perform and can be completed in less than about 15 minutes' time. The assays, devices and kits provide information which is useful, for example, in monitoring the impact of changes in such factors as diet, sleep, exercise, exposure to smoke or other carcinogens, and intake of alcohol or drugs on the fertility of sperm samples produced thereafter. Also, the devices, kits and methods can indicate whether a particular male fertility or contraceptive treatment has been effective or whether sperm subsequently obtained from the same male will initiate a pregnancy upon contact with an oocyte. In addition, the assays, devices and kits are useful for determining whether a particular sperm sample is suitable for use in an ART.

Certain of the assays, devices and kits are appropriate for use in a reference laboratory, while others can be used in a doctor's office and/or at home. Other features and advantages will become readily apparent from the following Detailed Description and Claims.

Brief Description of the Figures

Figure 1 is a perspective view of one embodiment of a sperm isolation device, which includes a tube for receiving and capturing motile sperm.

Figure 2 is a perspective view of another embodiment of a sperm isolation device, which includes a less dense fluid layer in contact with a more dense sperm-containing sample.

Figure 3 is a perspective view of yet another embodiment of a sperm isolation device, in which a porous membrane separates the sperm sample from a motile sperm collection area.

Figure 4 is a graph plotting the motility recovery after cryopreservation for sperm samples having a stress test score of greater than about 0.8 and samples having a stress test score of less than about 0.8.

Detail d Description

The invention pertains to easy to use devices that can rapidly recover motile sperm from a sperm containing sample (e.g. semen) and assays and kits, which employ the devices for use in identifying a high pregnancy potential sperm sample. The invention also pertains to assays and kits for screening males for infertility and for confirming whether a male contraceptive is effective or has taken effect.

Preferred sperm isolation devices are based on sperm motility and comprise a housing means for holding sperm, an isolation means selectively disposable within the housing means for isolating motile sperm and a means for testing the motile sperm to determine pregnancy potential. One embodiment is illustrated in Figure 1. A container 10 that has side walls 12, 14, 16 and 18 and a bottom portion 20 forming a sample well 21 for receiving a sample 30 containing ejaculate from a male donor. Although illustrated as cubical, the container 10 can have any selected shape or size suitable for the capture and retention of a sperm containing sample. A retainer 24 can be positioned within the sample well 21 of the container, and is preferably placed in fluid communication with the sample 30. Those of ordinary skill will recognize that the retainer 24 can be mounted to the floor portion 20 of the container 10 by a number of known methods, including the use of an adhesive, or can be supported within the container 10 by any suitable support means. Although illustrated as cylindrical, the retainer 24 can have any selected shape or size suitable for the isolation and retention of motile sperm, and can include commercially available instruments, such as tubes, capillaries, straws, pipettes, and other suitable receptacles having an internal conduit. Additionally, the container 10, although illustrated as having a substantially rectangular shape, can be configured to have any selected shape, such as a funnel-like configuration, to assist the male donor in collecting and capturing a sperm sample. The container 10 and retainer 24 can be made from any suitable biocompatible material, such as glass or plastic.

The illustrated sample 30 can contain both motile and non-motile sperm. According to a preferred practice, the retainer 24 is positioned within the well 21 or constructed to allow the motile sperm to migrate into the conduit 22 of the receptacle 24. The receptacle 24 captures the motile sperm, which are then analyzed to determine the pregnancy potential of the sperm sample. For example, after a sufficient period of time has elapsed to ensure that motile sperm present in the sample 30 have migrated into the retainer 24, the retainer can be removed from the container 10 and placed within a second container 31, e.g., a test tube, containing a means for testing the motile sperm to determine the pregnancy potential of the sample 34.

Alternatively, a detection mechanism 36 can be mounted within the conduit 22 of the retainer 24. The motile sperm that migrate into the conduit 22 preferably contact the detection mechanism 36. The detection mechanism preferably includes a suitable membrane or substrate containing a means for testing motile sperm to determine the pregnancy potential of the sperm containing sample.

Motile sperm can alternatively be isolated from non-motile cells in a sperm sample based on contact with a mechanical or fluid barrier or gradient. For example, as shown in Figure 2, a relatively dense sperm-containing sample can be contacted with a less dense fluid layer, which is preferably of a similar temperature, pH and salt concentration as the sperm-containing sample. In Figure 2 and in all subsequent figures, like parts are represented throughout with the same reference numerals plus a superscript prime. The illustrated container 10' includes a first fluid layer 40 and a second fluid layer 46. The first fluid layer 40 includes a sperm sample from the male donor and optionally an appropriate reagent for liquefying the semen sample, e.g. pronase or chymotrypsin. The layer 40 typically includes motile and/or non-motile sperm. The second fluid layer 46 is preferably a fluid having a density that is less than the density of the first fluid layer 40, thus creating a density gradient along the height of the container 10'. The decreasing density between layers 40 and 46 promote the migration of motile sperm from the sample fluid layer 40 into the collection fluid layer 46. This axial layered construction of the fluids 40 and 46 can be accomplished by selecting appropriate fluids that are emissible or partially emissible relative to one another.

According to a preferred practice, a sperm sample is placed within the container or is mixed with a reagent for liquefying ejaculate prior to introduction into the container 10'. A second fluid layer 46 having a lower density is then introduced into the container 10' and is separated from and disposed axially above the first fluid layer 40. After a sufficient amount of time, the motile sperm present in the sample layer 40 migrate into the second fluid layer 46 in response to the diminishing density gradient along the height of the container 10'. Thus, the motile sperm are isolated in the second fluid layer 46. After a selected period of time (e.g., five minutes) a sample of fluid from fluid layer 46 is removed, as by a pipette 50, and is introduced into a test tube 52 which holds a test solution 54. The solution preferably contains a hypotonic solution having peroxidase-conjugated anti-SOD and/or anti-GPx IgG polyclonal antibodies. A test strip containing bound sheep anti-SOD and/or anti-GPx IgG polyclonal antibodies is placed into the test solution, removed and then placed in a final solution containing a peroxidase substrate (e.g. hydrogen peroxide and DAB). In response to the presence of motile sperm, the test strip changes to a selected color. This color is matched to a color chart to determine the pregnancy potential of the sperm

sample. Alternatively, the pipette containing motile sperm can be directly applied to a test strip, which contains a sperm reagent.

According to an alternate embodiment, as illustrated in Figure 3, the second collection fluid layer 46 can be replaced with a porous membrane 60. The permeable porous membrane 60 introduces or presents a mechanical resistance to the migration of non-motile sperm from the sample layer 40' into or through the membrane 60. However, the porous membrane 60 facilitates the migration and hence the isolation of motile sperm in the isolation layer 10' by allowing motile sperm to pass between sample layer 40' and the membrane 60. The porous membrane 60 can be composed of any suitable biocompatible material that facilitates the passage of motile sperm without introducing damage thereto.

In another aspect, the invention relates to preferred methods for determining the pregnancy potential of a sperm sample. A sperm sample with "high pregnancy potential" as described herein has a greater than about 50% probability for initiating a pregnancy upon contact with an oocyte. Although a sperm sample having high pregnancy potential has an increased probability of initiating a pregnancy, contact of a high pregnancy potential sperm sample with an oocyte does not guarantee a successful pregnancy. Other factors, such as inability of an oocyte to decondense human sperm chromatin, defective oocyte DNA (e.g. due to age), two-cell embryo block or early embryo demise, may prevent the initiation of a pregnancy even by a high pregnancy potential sperm sample.

A sperm sample with "low pregnancy potential", on the other hand, has very little or no chance of initiating a pregnancy upon contact with an oocyte. Indication that a sperm sample provided by a particular male has low pregnancy potential does not indicate that subsequent sperm samples will also have low potential. Since the pregnancy potential of the sperm samples produced by a given male can change over time and can be influenced by such factors as diet, sleep, exercise, exposure to smoke or other carcinogens, or intake of alcohol or drugs, the pregnancy potential of a particular sperm sample is in general only accurate for a period of about 48 hours.

Preferred sperm containing samples (e.g. semen) for use in the disclosed assays are obtained from a human or animal (e.g. a bull, stallion, ram or other domesticated animal or an endangered animal). To ensure accuracy, tests are preferably performed on freshly collected ejaculate.

Preferred tests for identifying sperm samples with high pregnancy potential are lipid peroxidation tests, which measure an indicator of lipid peroxidation or a change in an indicator over a period of time under defined conditions. The value obtained is then

compared with a standard value for that particular indicator to determine the pregnancy potential of that sperm sample.

A stress test measures a change in a particular indicator of lipid peroxidation over a period of time in response to a stress (a stress test). A stress test score can be obtained by dividing a measured post-stress value of an indicator of lipid peroxidation by a measured pre-stress value. Examples of appropriate stresses or stressing agents for obtaining a test score include radiation (such as thermal (e.g. as described in Example 2), electromagnetic), freeze-thawing, oxidation or exposure to chemical agents (e.g. oxidizing agents such as the ferrous iron/ascorbate system described in Example 3).

Examples of lipid peroxidation indicators that change in response to a stress include:

Motility: When motility is used as an indicator of lipid peroxidation and the stress is thermal (e.g. incubation for at least about 1 hour at a temperature in the range of about 27-45^{o}C), a test score of greater than about 0.8 indicates high pregnancy potential. A test score of less than about 0.8 indicates low potential.

Lipid peroxidation breakdown products: Samples that have a relatively high post-stress value of lipid peroxidation breakdown products (such as lipid hydroperoxides, malonaldehyde, pentane and ethane) relative to pre-stress value (test score < 0.5) have a low probability of resulting in a successful pregnancy, while test scores of about 0.5 or greater increase the probability of initiating a pregnancy. The lipids can be analyzed spectrophotometrically. For example, lipid hydroperoxides can be extracted with hexane and detected at 233nm. Malonaldehyde can be measured at 532nm following reaction with the thiobarbituric acid (Tapel, A.L. et al., (1959) *Arch Biochem Biophys. 80*:326). Pentane and ethane can be measured by head-space gas chromatography.

Ratio of membrane phospholipids: Samples that oxidize at high rates have phosphatidylethanolamine (PE)/ phosphatidylcholine (PC) ratios < about 0.5, while samples that oxidize at low rates (test scores > about 0.8 as indicated by loss of motility) have PE/PC ratios of greater than about 0.7. Therefore, sperm with high pregnancy potential have PE/PC ratios greater than about 0.7, but less than about 1.5, sperm with some pregnancy potential have PE/PC ratios of greater than about 0.5 and less than about 0.7, and sperm

with low pregnancy potential have PE/PC ratios of less than about 0.5. PE and PC can be measured, for example, by high-performance thin-layer chromatography (Alvarez, J.G. et al., (1987) *J Liquid Chromatogr 10: 3557*).

Table 1
Pregnancy Potential Based on Test Score

Indicator	Stress	Test Score
Lipid peroxidation breakdown products (e.g. lipid hydroperoxides, malonadehyde, pentane, ethane)	oxidation, increased temperature	≥ 0.5 High Pregnancy Potential <0.5 Low Pregnancy Potential
PE/PC	oxidation	>0.7 - <1.5 = High Pregnancy Potential >0.5 - 0.7 = Some Pregnancy Potential <0.5 = Low Pregnancy Potential
Motility	increased temperature	≥ 0.8 = High Pregnancy Potential < 0.8 = Low Pregnancy Potential

Examples of lipid peroxidation indicators that can be directly measured as an indication of pregnancy potential include:

Oxidation of DNA: Samples that oxidize at high rates have higher levels of oxo-8-deoxyguanosine (oxo^8dG), which can be measured, for example, by high-pressure liquid chromatography (HPLC) using electrochemical detection (Fraga, C.G. et al., (1991) *Proc Natl Acad Sci 88*:11003.). Sperm with high pregnancy potential have less than about 20 fmol of oxo^8dG/ µg of DNA, while sperm with low pregnancy potential have greater than about 40 fmol of oxo^8dG/ µg of DNA.

Superoxide dismutase (SOD) activity: Solid phase-bound anti Cu/Zn-SOD antibodies and enzyme labeled anti-Cu/Zn-SOD antibodies can be used to detect SOD activity as described in Example 3. Sperm samples with SOD activities greater than or equal to about $9U/10^8$ cells have been shown to be associated with high pregnancy rates after IVF (i.e. have high pregnancy potential), while activities less than about $7U/10^8$ cells are associated with low pregnancy rates (i.e. low pregnancy potential).

Surface SOD immunofluorescence Samples that oxidize at high rates have low surface-SOD immunofluorescence and low total SOD activity. Surface-SOD immunofluorescence can be measured, for example, by flow cytometry using sheep anti-Cu/Zn-SOD IgG polyclonal antibodies and FITC-

conjugated rabbit anti-sheep secondary antibodies (Alvarez, J.G. 18th Annual Meeting American Andrology Society, Tampa, FL., 1993, abstract 170). Greater than about 300 FITC units is indicative of high pregnancy potential, while less than about 300 FITC units is indicative of low pregnancy potential.

Ratio of unsaturated fatty acids to saturated fatty acids Unsaturated fatty acids are prone to oxidation while saturated fatty acids are insensitive to this process. Determination of the ratio of the unsaturated fatty acid, docosahexaenoic acid (22:6) to the saturated fatty acid palmitic acid (16:0) or of other unsaturated fatty acids to saturated fatty acids can be used to predict pregnancy potential. Samples that oxidize at high rates will have low docosahexaenoic acid/palmitic acid ratios. Unsaturated/saturated fatty acids can be measured by gas chromatography following alkaline methanolysis with 1N sodium methoxide at 40C for 1 hour (Alvarez, J.G. and J.C. Touchstone, Practical Manual on Lipid Analysis. Series of Monographs: I- Fatty Acids. Norell Press (New Jersey), 1991). A ratio of unsaturated to saturated fatty acids of greater than or equal to about 1.0 indicates high pregnancy potential; a ratio greater than or equal to about 0.5 and less than about 1.0 indicates borderline pregnancy potential; and a ratio of less than about 0.5 indicates low pregnancy potential.

Creatine kinase activity The concentration of creatine kinase in sperm reflects the degree of cytoplasmic extrusion during the last phase of spermatogenesis. Samples exhibiting abnormally high levels of creatine kinase activity have also been found to have high rates of lipid peroxidation as determined by the PE/PC ratios (PE/PC ratios < 0.5 and increased malonaldehyde production). This correlation supports the detection of creatine kinase activity as a means for determining pregnancy potential of a sperm sample. Creatine kinase activity in human sperm can be measured by spectrophotometric analysis at 365 nm of the NADPH generated during reaction of creatine kinase with ADP to produce creatine and ATP, followed by reaction of hexokinase with ATP and glucose to produce glucose-6-phosphate, followed by reaction of glucose-6-phosphate dehydrogenase with glucose-6-phosphate and NADP to produce NADPH. The NADPH generated under these conditions is proportional to the activity of creatine kinase in human sperm (Huszar, G. (1988) *Gamete Res 19*:67.). Creatine kinase (CK) exists in two isoforms: MM and BB. Sperm with high pregnancy potential have a CK-MM/CK-MM-CK-BB ratio of greater than or equal to about 10%.

Table 2
Pregnancy Potential or Contraceptive Efficacy Values from Direct Indicators of Lipid Peroxidation

Indicator	High Pregnancy Potential	Low Pregnancy Potential
SOD Activity	≥ 9 U/10^8	<7U/10^8
oxo^8dG	<20 fmol/ µg DNA	>40 fmol/ µg DNA
Surface SOD Immunofluorescence	>300 FITC	<300 FITC
Unsaturated/Saturated Fatty Acid Content	≥ 1.0	< 0.5
CK-MM/CK-MM + CK-BB	$\geq 10\%$	$< 10\%$

Lipid peroxidation indicator values or changes in value in response to a stress can be quantitated by techniques and devices, which are well-known to one of skill in the art. The particular lipid peroxidation indicator chosen may be dictated by the degree of accuracy required and the availability of instruments to detect the results.

In a preferred embodiment for use as a kit, which is described in detail in the following Example 4, the indicator of sperm lipid peroxidation is based on sperm superoxide dismutase (SOD) activity. In a particularly preferred embodiment, sperm SOD activity is tested using an SOD antibody. As used herein, an antibody for use in detecting a sperm lipid peroxidation indicator can be any material that binds an antigen (e.g. polyclonal, monoclonal or single chain antibody or antibody fragment, such as an Fab of Fab'2 fragment).

Immunodetection of sperm SOD or another antigenic indicator of lipid peroxidation can be accomplished using any of a number of competitive or non-competitive assay procedures. In general competitive immunoassays are performed by adding SOD to a sperm containing sample, so that the sperm and the SOD compete for a limited number of antibody binding sites resulting in the formation of sperm-antibody and labeled SOD-antibody complexes. By maintaining the concentration of labeled SOD and SOD antibody constant, the amount of labeled SOD-antibody complex formed is inversely proportional to the amount of sperm present in the sample. A quantitive determination of the sperm SOD can therefore be made based on the labeled SOD-antibody complex. Competitive assays can be homogeneous (i.e. not requiring separation of antibody bound tracer (e.g. labeled SOD) from free tracer, since the antigen-antibody interaction causes, directly or indirectly, a measurable change in the signal obtained from the label group of the tracer). Alternatively, competitive assays can be heterogeneous (i.e. requiring separation of bound tracer from free tracer prior to determining the amount of ligand in the sample).

In contrast to competitive immunoassays, non-competitive assays involve incubating a sperm containing sample with an immobilized SOD antibody for a period of

time sufficient to reach equilibrium with regard to the formation of antibody-sperm conjugates. The SOD antibody can be directly or indirectly labeled. For example, indirect labeling can be carried out after a wash step to remove unbound sperm by contacting the immobilized antibody-sperm complexes with a second, labeled antibody that is specific for the antibody-sperm complex. Following a second wash step to remove unbound second antibody, the amount of bound second antibody can be detected and measured as an indication of bound sperm. An example of this procedure is described in the following Example 5.

Exemplary competitive and non-competitive immunoassays include fluorescence polarization immunoassay (FPIA), fluorescence immunoassay (FIA), enzyme immunoassay (EIA), nephelometric inhibition immunoassay (NIA), enzyme linked immunosorbent assay (ELISA) and radioimmunoassay (RIA). General techniques for performing the various immunoassays are known to one of skill in the art. Moreover, a general description of most procedures is provided in U.S. Patent No. 5,051,361, which is incorporated herein by reference. The antibodies can be labeled in any manner that facilitates detection. Preferred labels include enzymes (e.g. horseradish peroxidase, alkaline phosphatase, urease, β-galactosidase), enzyme co-factors, radioisotopes (e.g. ^{3}H, ^{14}C, ^{125}I, ^{32}P, ^{131}I and ^{35}S), fluorescent compounds (e.g. fluorescein, rhodamine, allophycocyanin, phycoerythin, erythrosin, europian, luminol, luciferin and coumarin) and colored or uncolored beads or particles (e.g. silica gel, controlled pore glass, magnetic, Sephadex/Sepharose, cellulose, metal (e.g. gold) or latex). Preferred supports for immobilizing antibodies include membranes (e.g. polyethylene, polypropylene, polyamide, polyvinylidenedifluoride, glass fiber, paper), beads or particles and tubes, (e.g., glass, plastic or metal capillaries, straws or pipettes).

Based on the above-described procedures for identifying sperm samples with high pregnancy potential, the invention also features processes for increasing the success rate for initiating a preganancy using an assisted reproductive technology (ART) (i.e. a procedure for contacting a sperm with an ovum to initiate a pregnancy). Examples of ARTs include in vitro fertilization (IVF), gamete intrafallopian transfer (GIFT) intrauterine insemination (IUI) and intracytoplasmic sperm injection (ICSI). Procedures for performing ARTs are well-known to practitioners. It is expected that additional ARTs will be developed over time.

A preferred process of the invention involves obtaining multiple sperm samples from a donor (e.g. a male partner from a couple undergoing an ART) over time. For example, a donor can provide a new sperm sample once every other day. An aliquot (e.g. 1×10^6 cells) of each sample can then be obtained for testing, while the remainder can be banked (e.g. cryopreserved) for potential future use in an ART. Preferably the process includes a

means for correlating a particular aliquot with the banked sample from which it was obtained. For example, an aliquot taken from a sample, as well as the sample itself obtained on day 1 can be labelled #1. A subsequently obtained sample from the same donor and an aliquot taken from that sample can be labelled #2, etc..

All aliquots provided by a particular donor can then be tested to identify sperm samples having "high pregnancy potential" (e.g. sperm having a greater than 50% probability of initiating a pregnancy upon contacting an oocyte). The sperm sample indicated as having the higher pregnancy potential can then be used in an ART and the remaining, lower pregnancy potential samples can be discarded.

The following Example 2 provides the results of a blind prospective cohort study of 33 couples undergoing in vitro fertilization (IVF) and 11 couples undergoing gamete intrafallopian transfer (GIFT), no successful pregnancies resulted from sperm samples which exhibited a stress test score of less than about 0.8. In contrast, when sperm samples having a stress test score of about 0.8 or greater were used in an ART, a pregnancy resulted 55% of the time. Therefore, where the indicator of lipid peroxidation is loss of motility, a stress test score of less than about 0.8 indicates low pregnancy potential, while a stress test score of about 0.8 or above indicates high pregnancy potential and is suitable for use in an ART.

Table 3 shows how the stress test score and therefore the pregnancy potential of sperm obtained from the same male can vary with time.

Table 3

ST scores for IVF male partners

Patient	Date of IVF	ST score
1.	11/05/93	0.35
	04/20/94	0.88
2.	11/15/93	0.88
	09/27/94	0.88
3.	11/17/93	0.88
	06/28/94	0.88
4.	01/24/94	1.00
	02/16/94	0.50
5.	01/05/94	0.20
	06/20/94	0.92
6.	05/03/94	1.00
	06/29/94	0.40
7.	05/05/94	1.00
	06/27/94	0.50
8.	02/22/94	0.80
	10/05/94	1.00
9.	06/28/94	1.00
	10/18/94	1.00

In addition, Figure 4 shows that sperm samples with stress test scores greater than about 0.8 have a high motility recovery, even after cryopreservation. Therefore it appears that a further benefit of using sperm samples indicated as having high pregnancy potential in an ART results from the fact that such samples are not damaged by cryopreservation to the same degree as sperm with low pregnancy potential.

In contrast to high pregnancy potential sperm samples, identification of sperm samples with low pregnancy potential can also be useful, for example, as a screen for detecting potentially infertile males. In addition, since even fertile males can occassionally generate ejaculates with low pregnancy potential, by identifying a sperm sample as having low pregnancy potential, use of the sample in an ART can be avoided. Further, by identifying sperm samples with low pregnancy potential, the user can determine whether a particular male contraceptive is effective or has taken effect. For example, a vasectomy typically requires a phase in period in which to take effect. The use of assays to identify

sperm samples with low pregnancy potential, as disclosed herein, is therefore useful for confirming the efficacy of a male contraceptive.

Low pregnancy potential sperm samples can be identified by lipid peroxidation tests as described above. In addition, low pregnancy potential sperm samples can be identified based on quantification of spermatozoa (non-motile and motile) in a sperm containing sample (e.g. ejaculate). If the number obtained is less than about 20 million spermatozoa/mL, the sperm sample is considered to have low pregnancy potential.

Examples of sperm targets or sperm components that can be quantitated as an indication of the number of sperm present in a sperm containing sample, include a sperm protein (e.g. a sperm flagella protein, a glycolytic enzyme, an antioxidant enzyme (e.g. glutathione peroxidase or superoxide dismutase), a nuclear protein, an acrosomal protein, α-tubulin, lactate dehydrogenase (LDH-X), protamine, acrosin or a mitochondrial protein.); or a sperm lipid (e.g. cholesterol, a phospholipid, a glycolipid, a triglyceride, a fatty acid, phosphatidylglycerol, seminolipid, and a docosahexaenoic acid). Preferred targets for sperm quantitation are selective for and abundant on sperm cells.

Preferably a sperm reagent (e.g. sperm antibody, ligand, lectin or substrate) is used to quantitate sperm. Preferred sperm reagents include labeled (e.g. enzyme, tracer (e.g. radioactive), dye or color particle labeled) or unlabeled anti-sperm antibodies (e.g. anti-human sperm polyclonal antibody; Arnel Products Co., Inc. Cherokee Station, New York, N.Y.; Chemicon International Inc., Temecula, California) or labeled or unlabeled antibodies against a sperm component (e.g. a sperm protein or sperm lipid). Further preferred sperm reagents include labeled (e.g. dye or tracer labeled) or unlabeled reagents that interact with a sperm protein (e.g. Protein Reagent (0.3% tetrabromophenol, Miles Scientific, Connecticut) or rhodamine 123, which accumulates within sperm mitochondria. Alternatively, the labeled reagent (e.g. rhodamine) can interact with a sperm lipid.

Preferred methods for counting sperm employ detectably labeled antibodies that specifically bind a sperm antigen (e.g. anti-human sperm polyclonal antibodies and antibodies specific to an epitope of the sperm flagellum, nuclear proteins, glycolytic enzymes, acrosome etc.). One method for quantitating sperm involves incubating a sperm sample with colored particles containing anti-sperm antibodies for an appropriate period of time to allow the sperm antigens to react with the antibody bound colored particles; ii) filtering the sample of step i), so that sperm/colored particle/antibody complex is retained on the filter and unbound, colored particle/antibody and seminal plasma protein passes through the filter; and iii) visualizing the color intensity.

For example, if colored particles are used, sperm/colored particle/antibody complexes can be quantitated by comparing the color of the filter to a color chart, which depicts various color possibilities for various quantities of sperm. Less than about 20 million spermatozoa/mL indicates low pregnancy potential. Alternatively, the filter membrane can be configured to indicate a "+" (i.e. fertility), if a sufficient amount of sperm/detectable particle/antibody complex is retained on the filter membrane to indicate that the sample is suitable for initiating a pregnancy (greater than about 20 million spermatozoa/mL) and a "-" (i.e. infertility) if an insufficient amount of sperm/detectable particle/antibody complex is retained on the filter membrane (less than about 20 million spermatozoa/mL).

In order to use antibodies or reagents that may also react with seminal plasma components present in a sperm containing sample (e.g. ejaculate), sperm can first be isolated. Seminal plasma-free sperm can then be contacted with anti-sperm antibody coated colored particles. After a sufficient period of time to allow antibodies and antigens to react, unbound antibody coated colored particles can be removed from the mixture and sperm/colored particle/antibody complex detected and quantitated. For example, the quantity of sperm in the sample can be quantitated using a color chart or a "+" or "-" filter as described above. Alternatively, sperm can be quantitated by detecting the appearance of agglutination in a drop of sample following addition of anti-sperm antibodies with bound latex particles.

Alternatively, low pregnancy potential can be determined by quantitating only motile sperm in a sperm sample. Motile sperm can be isolated from a sperm containing sample, for example, using the devices and procedures described above. Preferred methods for quantitating sperm employ detectably labeled antibodies that specifically bind a sperm antigen. Motile sperm can be quantitated using detectably labeled, sperm specific antibodies, such as anti-glutathione peroxidase (GPx) antibody. As with sperm SOD, immunodetection of sperm GPx or other sperm antigen can be accomplished using any of a number of competitive or noncompetitive assay procedures. In addition, sperm specific antibodies can be labeled (e.g. enzymatically) and if necessary immobilized by procedures that are well known in the art or as described above for SOD antibodies.

In a further aspect, the invention features sperm diagnostic kits or systems comprising a number of simple reagents and devices packaged in a box. The kit components can include one or more of the devices or components illustrated in Figures 1-3, and reagents for determining the pregnancy potential of sperm samples. Preferable kits include reagents for liquefying semen (e.g. chymotrypsin or pronase). For identifying sperm samples with high pregnancy potential (i.e. Male Fertility kit), preferable kits are based on measuring an indicator of lipid peroxidation or a change in an indicator over a period of time under defined conditions. Alternatively, as further described in the following Examples, a kit can be based

on determining the number of spermatozoa present in a sperm sample, wherein greater than about 40 million motile spermatozoa/mL indicates high pregnancy potential; less than about 20 million motile spermatozoa/mL indicates low pregnancy potential; and greater than about 20 million, but less than about 40 million indicates borderline pregnancy potential.

Preferable kits for identifying a sperm sample with low pregnancy potential, (e.g. to confirm the effectiveness of male contraception) are based on determining the number of spermatozoa/mL present in the sample, wherein less than about 50,000 spermatozoa/mL indicates low pregnancy potential or successful contraception. Reagents and devices for use in determining sperm pregnancy potential can include dispensing devices (e.g. capillaries or pipettes) for delivering a defined volume of isolated sperm from a collection container into a container which includes a means for determining the pregnancy potential of the sample, for example by quantitating the extent of sperm lipid peroxidation or change in lipid peroxidation resulting from a stress; and/ or sperm count.

In a preferred embodiment, the means for detecting sperm lipid peroxidation is based on sperm superoxide dismutase (SOD) activity and/or the means for detecting sperm count is based on sperm protein concentration. In a particularly preferred embodiment, the means for detecting sperm SOD activity employs a colorimetrically labeled SOD antibody and the means for detecting sperm protein employs gold particles. The kit also preferably includes a color chart against which concentrations of SOD and/or protein can be compared against known values. The kit can be optimized to develop color based on the protein levels corresponding to sperm concentrations greater than or equal to about 50,000 spermatozoa/mL.

A preferred kit for home determination of the pregnancy potential of a sperm sample can include: i) a collection container (optionally conformed to capture ejaculate, and/or containing a solution (e.g. enzymatic) that facilitates semen liquefaction); ii) a second container in which is contained anti-sperm antibodies covalently bound to colored particles in a hypotonic solution; iii) a membrane or filter for capturing sperm/colored particle or sperm/colored particle/antibody complexes and allowing passage of unbound, colored particle or colored particle/antibody; and iv) a chart for interpreting the results obtained based on the color of the filter. Indication of less than about 20 million spermatozoa/mL indicates low pregnancy potential when using the Male Fertility approach; or less than about 50,000 spermatozoa/mL when using the Male Contraceptive approach.

The present invention is further illustrated by the following Examples which are intended merely to further illustrate and should not be construed as limiting. The entire contents of all of the references (including literature references, issued patents, published

patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

Example 1: Kit for Isolating Motile Sperm From a Semen Sample

Kit Components:

1) (1) container (tube #1) for collecting and liquefying ejaculate containing 30mg/mL of dextran and 5mg/mL of chymotrypsin in an isotonic solution;

2) (1) see-through container (tube #2), which indicates three volumes: lower (1 mL), middle (1.5 mL) and upper (2 mL). The three volumes can be indicated by black and red marks (the top and the bottom mark being of the same color);

3) 0.5 mL container of isotonic solution (tube #3)

3) (1) 0.5 mL container of bovine serum albumin (BSA) at 0.2 mg/mL in an isotonic solution (tube #4);

4) (1) empty container;

5) (at least 3) disposable sterile pipettes or capillaries;

6) (1) capillary pipette to remove and transfer motile sperm

7) (at least 1) test strip containing a means for quantitating sperm (e.g. protein reagent);

8) instructions for use

9) (1) heating block at 37°C (optional)

Procedure:

The ejaculate was collected in the collection tube (tube #1) containing 5mg/mL of chymotrypsin and 30mg/mL of dextran in an isotonic solution. The mixture was allowed to stand at room temperature for about 5 minutes.

Using a dropper, 1 mL aliquots of the semen/dextran/chymotrypsin mixture were added to the calibrated see-through container (tube #2), so that the mixture reached the first marked level. The pipette was disposed of.

Using a second dropper, an appropriate volume of the isotonic solution (container #3) was used to overlay the semen/dextran/chymotrypsin mixture to the upper mark of each see-through container, resulting in the formation of a two layer density gradient: the lower layer consisting of the semen/dextran/chymotrypsin mixture and the upper layer consisting of the isotonic solution. The dropper was disposed of.

The mixture was allowed to stand for at least 5 minutes at room temperature or optionally in a 37°C heating block.

A sterile capillary pipette was inserted to reach the middle mark of the container and applied to a test strip which contained a means for quantitating sperm, e.g., protein reagent, lipid reagent.

Example 2: Temperature Stress for Predicting Pregnancy Potential of a Sperm Sample

Human sperm (HS) samples were obtained from 44 male partners of couples undergoing in vitro fertilization (IVF) (n=33) or gamete intrafallopian transfer (GIFT) (n=11). All male partners had normal semen by conventional analysis (By convention, a normal semen sample is defined as having greater than 20 million cells/mL, greater than 60% motility, a rate of progression greater than 2 (on a scale of 1 to 4), greater than 60% normal forms, 1.5 to 5.0 mL of ejaculate, no significant sperm agglutination, no significant number of leucocytes, and no hyperviscosity). Sperm samples were washed using the standard Percoll procedure and resuspended in HTF medium containing 10% plasmanate. An aliquot of the sperm suspension was utilized for artificial reproductive technologies (ART, e.g. IVF and GIFT) and a 100µL aliquot of the same sperm suspension was incubated at 40°C for 4h (stress test). Stress test scores were expressed as the ratio of final to initial motility. Analysis was performed by stepwise multiple regression. The independent variables were: women's age; HS motility before and after the stress test; stress test score; and the number of embryos transferred per IVF cycle. The dependent variables were: pregnancy and fertilization outcome.

In a blind cohort study, of the 44 ART cycles, 11 (25%) resulted in pregnancy. Of the 44 sperm samples used in the study, 24 (55%) had stress test scores < 0.8 and 20 (45%) > 0.8. Controlling for all variables, the stress test score was significantly correlated with pregnancy outcome (P = 0.0001). In sharp contrast, the motility before the stress test was not correlated with pregnancy outcome. All pregnancies occurred in sperm samples with stress test scores > 0.8. Therefore, a cut-off value > 0.8 was selected to predict pregnancy outcome. The sensitivity of the stress test was 100% and the specificity was 73%. The negative predictive value of the test, defined as the absence of pregnancy with stress test scores < 0.8, was 100% and the positive predictive value, defined as the occurrence of pregnancy with stress test scores > 0.8, was 55%. Although the stress test score is highly predictive of pregnancy outcome, the fertilization rate was better predicted by the motility after the stress test (P = 0.007).

Example 3: Oxidative Stress for Predicting Pregnancy P tential of a Sperm Sample

A similar test as described in Example 4 was carried out, except that the stress was provided by adding 10µL of 0.125mM ferrous iron in PBS (phosphate based saline) and 0.6mM ascorbate also in PBS to 30µL sperm suspensions for a total volume of 50 µL and incubating the mixture for 0.5h at 37°C with gentle shaking. Results indicate that the values obtained correlate with the stress test score at 40°C for 4h.

Example 4: Superoxide Dismutase (SOD) Activity in Sperm Correlates with Pregnancy Rate

Human spermatozoa release oxygen radicals and undergo spontaneous lipid peroxidation resulting in extensive damage to the sperm plasma and acrosomal membranes and loss of their pregnancy potential. Cu/Zn-superoxide dismutase (SOD) protects sperm against oxygen radical-mediated toxicity and endogenous lipid peroxidation. Surface SOD immunoreactivity is expressed over the mid piece and post equatorial regions of human sperm (Alvarez and Storey, (1992) *J. Andrology* 13(3):232-241, the contents of which are incorporated herein by reference).

Whether SOD activity correlates with sperm pregnancy potential and pregnancy rate was studied using sperm samples obtained from 27 male partners from couples undergoing in vitro fertilization (IVF). The age of the female partner ranged from 24 to 49 years. The number of oocytes inseminated ranged from 3 to 20 and the number of embryos transferred from 1 to 7. Sperm samples were washed using the standard mini Percoll procedure, and resuspended in HTF medium containing 10% plasmanate. An aliquot of the sperm suspension was utilized for IVF and another aliquot was used to measure the SOD activity of that particular sample.

SOD activity in the sperm samples examined ranged from 1.5 to 12 U/10^8 cells ($P < 0.001$). All six sperm samples with SOD activities less than or equal to 3.3U/10^8 cells resulted in failed fertilization ($P < 0.001$). Between 4.7 and 7.1U/10^8 cells, fifteen out of sixteen oocytes were fertilized but no ongoing pregnancies resulted.

These results show that sperm samples with high SOD activity (i.e. greater than about 9 units/ 10^8 cells) have a high pregnancy potential, while samples with low SOD activity (i.e. less than about 7 units/ 10^8 cells) have a low pregnancy potential. Samples having an SOD activity in the range of greater than or equal to about 7 and less than about 9 units/10^8 cells have borderline pregnancy potential.

Example 5: Assay for Identifying a High Pregnancy Potential Sperm Sample

The ejaculate was collected in the collection tube (tube #1) containing 5mg/mL of chymotrypsin. After about three to five minutes (at which time the semen should be liquefied), an aliquot (about 50μL) of the liquefied semen was added to another test tube (tube #2), which contained horseradish peroxidase-conjugated sheep anti-human superoxide dismutase (SOD) IgG polyclonal antibodies (The Binding Site, Inc., 5889 Oberlin Drive, San Diego, CA 92121) and horseradish peroxidase-conjugated sheep anti-human glutathione peroxidase (GPx) IgG polyclonal antibodies (The Binding Site, Inc., 5889 Oberlin Drive, San Diego, CA 92121) dissolved in a hypotonic solution. After five minutes, a dip test strip with bound sheep anti-human SOD and anti-human GPx Ig G polyclonal antibodies (The Binding Site, Inc., 5889 Oberlin Drive, San Diego, CA 92121) was inserted into test tube#2. After five minutes, the test strip was removed from the tube and rinsed with tap water.

The test strip was then dipped into another test tube (tube #3), which contained the peroxidase substrate, hydrogen peroxide (H_2O_2) and diaminobenzidine (DAB); (Sigma Chemical Co., St. Louis, MO 63178). The color ratio of SOD/GPx provides an indication of pregnancy potential by comparison with a chart provided with the kit. Color in the GPx pad indicates equal or greater than about 20 million spermatozoa/mL. If the color in the SOD pad is higher than the color in the GPx pad, a high pregnancy potential of the sperm is indicated. If the color in the SOD pad is equal to or lower than the color in the GPx pad, a low pregnancy potential is indicated.

Example 6: Assay for Monitoring the Efficacy of Male Contraception (Color Chart Approach)

The ejaculate from a male who had taken an appropriate dose of a male contraceptive that suppresses spermatogenesis (e.g. a GnRH antagonist) or who had had a vasectomy, was collected in the collection tube (tube #1) containing 5mg/mL of chymotrypsin in an isotonic solution. After three to five minutes (at which time the semen should be liquefied) about 500 μL of the liquefied semen was added to another test tube (tube #2) containing anti-human sperm polyclonal antibodies (Arnel Products Co., Inc., Cherokee Station, New York, N.Y.; Chemicon International Inc., Temecular, CA) covalently bound to gold particles (having an intrinsic pink color) or to colored latex particles (having a blue or yellow color) in a hypotonic or isotonic solution. The mixture was incubated for up to about 10 minutes to allow the sperm antigens exposed after the hypotonic treatment to react with the antibodies. Following incubation, the contents of test tube #2 was transferred to a filter membrane, so that sperm/colored particle-antibody complex was retained and unbound.

colored particle/antibody passed through the filter into a reservoir. The color of the filter can then compared to various color possibilities depicted on a chart that is included with the kit to determine the number of sperm present in the sample and thereby determine the efficacy of the male contraceptive treatment. This approach allows one to detect in semen a concentration of at least 50,000 spermatozoa/mL. This sensitivity is comparable to that obtained with the regular light microscope commonly used in andrology laboratories.

Example 7: Assay for Monitoring the Efficacy of Male Contraception (Plus (+) or Minus (-) Approach)

The ejaculate was collected in the collection tube (tube #1) containing 5mg/mL of chymotrypsin in an isotonic solution. After about 3 to 5 minutes (at which time the semen should be liquefied) a drop (50µl) (Male Fertility Kit) or 0.5 mL (Male Contraceptive Kit) of the liquefied semen was added to another test tube (tube #2) containing a hypotonic solution. After a period of incubation of 5 minutes, a drop (about 50µL) (Male Fertility Kit) or the total contents (Male Contraceptive Kit) was transferred to a membrane provided with two windows: window #1 was used to apply the sample where after the sample was applied, the soluble sperm antigens reacted with anti-human sperm antibodies conjugated to gold or colored latex particles included in a reservoir in the membrane; window #2 had a horizontal colored line. As the sperm antigen/antibody/particle complex migrated through the membrane, a second anti-human sperm antibody bound in the vertical position to the membrane underlying window #2 (capture antibody) reacted with the sperm antigen/antibody/particle complex producing a colored vertical line when the sperm concentration in semen was above 50,000 spermatozoa/mL in which case a plus (+) sign appeared. If the concentration of sperm in semen was below 50,000 spermatozoa/mL, a minus (-) sign appeared.

Example 8: Male Fertility & Contraceptive Screening Kits (Filter Approach)

Kit Components

1) (1) calibrated semen collection tube:
2) (1) squeeze bottle containing the chymotrypsin solution:
3) (1) test tube containing the isotonic or hypotonic solution:
4) (2) droppers:
5) (1) filter mounted on a liquid reservoir:
6) (1) squeeze bottle containing anti-sperm antibody-coated colored latex or gold particle solution:
7) instructions for use.

Procedure

Using the squeeze bottle, chymotrypsin solution (dextran-free) was added to the semen collection tube in a volume equal to the semen volume. The mixture was allowed to stand at room temperature for about 5 minutes in order for the semen to liquefy.

Using a dropper, one drop (Male Fertility Kit) or 0.5 mL (Male Contraceptive Kit) of the semen/chymotrypsin mixture in the semen collection tube was added to another test tube (test tube #2) containing an isotonic solution (e.g. phosphate buffered saline or Earle's Medium) or a hypotonic solution (e.g. distilled water) .

One drop (50µL) (Male Fertility Kit) or 0.5 mL (Male Contraceptive Kit) of test tube #2 was added to a filter (e.g. blocked nitrocellulose, 5µm pore size or microfiber glass filter, 2.7 µm pore size) mounted on a liquid reservoir. Seminal plasma proteins, which were about 10-20 nm in size passed through the filter, while sperm cells which were about 1000 times bigger (50µm in size) were retained.

One drop of anti-sperm antibody-coated colored particles (e.g. gold particles that have an intrinsic pink color; or a colored latex particle) was added onto the filter and the appearance of color on the filter indicated a potentially fertile sperm sample. By using this filter procedure, the antibody used to coat the latex particle did not have to be highly specific for the sperm cell (i.e. it could exhibit some cross-reactivity with seminal plasma proteins).

Example 9: Male Fertility & C ntraceptive Screening Kits (Filter Approach with Preincubation of the Antibody and the Sperm Antigens)

Kit Components

1) (1) calibrated semen collection tube;
2) (1) squeeze bottle containing the chymotrypsin solution;
3) (1) test tube containing the isotonic or hypotonic solution;
4) (2) droppers;
5) (1) filter mounted on a liquid reservoir;
6) (1) squeeze bottle containing anti-sperm antibody-coated colored latex or gold particle solution;
7) instructions for use.

Procedure

Using the squeeze bottle, chymotrypsin solution (dextran-free) was added to the semen collection tube in a volume equal to the semen volume. The mixture was allowed to stand at room temperature for about 5 minutes in order for the semen to liquefy.

Using a dropper, one drop (about 50µL) (Male Fertility Kit) or 0.5 mL (Male Contraceptive Kit) of the semen/chymotrypsin mixture in the semen collection tube was added to another test tube (test tube #2) containing anti-sperm antibody-coated colored particles (e.g. gold particles that have an intrinsic pink color; or a colored latex particle) in an isotonic solution (e.g. phosphate buffered saline or Earle's Medium) or hypotonic solution (e.g. distilled water) .

One drop (50µL) (Male Fertility Kit) or 0.5 mL (Male Contraceptive Kit) of the contents of test tube #2 was added to a filter (e.g. nitrocellulose, 5µm pore size or microfiber glass filter, 2.7 µm pore size) mounted on a liquid reservoir. Seminal plasma proteins/color particle antibody/complex, which were about 10-20 nm in size passed through the filter, while sperm cells/colored particle antibody/complex which were about 1000 times bigger (50µm in size) were retained. By using this filter procedure, the antibody used did not have to be highly specific for the sperm cell (i.e. it may show some cross-reactivity with seminal plasma proteins).

Example 10: Male Fertility & Contraceptive Screening Kits (Agglutination Approach)

Kit Components

1) (1) calibrated semen collection tube;
2) (1) squeeze bottle containing the chymotrypsin solution;
3) (1) test tube containing an isotonic or hypotonic solution and sperm-specific antibody-coated particles (uncolored or colored);
4) (2) droppers;
5) (1) slide having a color, which contrasts with the anti-sperm antibody-coated particles.
6) instructions for use.

Procedure

Using the squeeze bottle, chymotrypsin solution (dextran-free) was added to the semen collection tube (tube #1) in a volume equal to the semen volume. The mixture was allowed to stand at room temperature for about 5 minutes in order for the semen to liquefy.

Using a dropper, one drop (50µL) (Male Fertility Kit) or 0.5 mL (Male Contraceptive Kit) of the semen/chymotrypsin mixture was added to another test tube (test tube #2) containing an isotonic solution (e.g. phosphate buffered saline or Earle's Medium) or hypotonic solution (e.g. distilled water) and sperm-specific antibody-coated particles (uncolored or colored).

One drop of test tube #2 was deposited on a slide, which was of a color that contrasted with the sperm specific antibody coated latex particles, so that agglutination, indicating the potential presence of fertile sperm could be discerned on the slide. The absence of agglutination when using the Male Fertility approach, was indicative of less than about 20 million spermatozoa/mL; borderline agglutination was indicative of greater than about 20 million spermatozoa/mL and less than about 40 million spermatozoa/mL; and the presence of high agglutination was indicative of greater than about 40 million spermatozoa/mL. The absence of agglutination when using the Male Contraceptive approach was indicative of less than about 50,000 spermatozoa/mL.

Example 11: Male Fertility & Contraceptive Screening Kits (Non Immunoreactive/Filter Approach)

Kit Components

1) (1) calibrated semen collection tube;
2) (1) squeeze bottle containing the chymotrypsin solution;
3) (1) test tube containing the isotonic or hypotonic solution;
4) (2) droppers;
5) (1) filter mounted on a liquid reservoir;
6) (1) squeeze bottle containing colored reagents that react non-specifically with sperm cell components, e.g., pink-colored gold particles (reacts with proteins) or red-colored rhodamine (reacts with lipids).
7) instructions for use.

Procedure

Using the squeeze bottle, chymotrypsin solution (dextran-free) was added to the semen collection tube (tube #1) in a volume equal to the semen volume. The mixture was allowed to stand at room temperature for about 5 minutes in order for the semen to liquefy.

Using a dropper, one drop (50µL) (Male Fertility Kit) or 0.5 mL (Male Contraceptive Kit) of the liquefied semen was added to another test tube (tube #2) containing gold particles (that have an intrinsic pink color) in a hypotonic or isotonic solution. One drop (Male Fertility Kit) or 0.5 mL (Male Contraceptive Kit) of the contents of tube #2 was added onto the filter (nitrocellulose with 5µm pore size or microfiber glass filter with a pore size of 2.7µm). Gold particles have an intrinsic pink color and high affinity for biomolecules (e.g. proteins or lipids) and therefore can bind to both spermatozoa and seminal plasma proteins. By using this filter procedure, the unbound gold particles and the gold particles bound to seminal plasma proteins went through the filter into the reservoir while sperm cells with bound gold particles were retained on the filter indicating a pink color. The presence of a pink color when using the Male Fertility approach, was indicative of the presence of greater than about 20 million spermatozoa/mL; and greater than about 50,000 spermatozoa/mL when the Male Contraceptive approach was used.

Example 12: Assay for Identifying a High Pregnancy Potential Sperm Sample

The ejaculate was collected in the collection tube (tube #1) containing 5mg/mL of chymotrypsin in an isotonic solution. After about 3 to 5 minutes (at which time the semen should have been liquefied) a drop (about 50μL) of the liquefied semen was added to a test tube (tube #2) containing anti-glutathione peroxidase (GPx) antibodies bound to yellow colored particles and anti-superoxide dismutase (SOD) antibodies bound to blue colored particles in a hypotonic or isotonic solution. The mixture was incubated for up to about 10 minutes to allow the sperm antigens exposed after the hypotonic treatment to react with the antibodies. Following incubation, the contents of test tube #2 was transferred to a filter membrane, so that sperm/colored particle/antibody complex was retained and unbound, colored particle/antibody passed through the filter into a reservoir. If the filter appeared green, the SOD/GPx ratio was about 1:1 and the sample had borderline pregnancy potential; if the filter appeared bluish, high SOD content was indicated and the sample had high pregnancy potential; and if the filter appeared yellowish, low SOD content was indicated and the sample had low pregnancy potential.

Example 13: Male Fertility & Contraceptive Screening Kit

Kit Components

1) (1) calibrated semen collection tube;
2) (1) squeeze bottle containing the liquefying enzyme (e.g. chymotrypsin or pronase at 5 mg/ml.) (squeeze bottle #1);
3) (1) squeeze bottle (with a removable top) containing 150μL of an isotonic or hypotonic solution (squeeze bottle #2);
4) (1) squeeze bottle containing a 0.5 mg/mL solution of Rhodamine-123 (Molecular Probes, Inc. Eugene, OR) in distilled water (squeeze bottle #3);
5) (1) dropper;
6) (1) porous filter mounted on a liquid reservoir;
7) instructions for use.

Procedure

Using the squeeze bottle provided (squeeze bottle #1), two drops (per milliliter of semen) of a solution of pronase solution (5mg/mL) (Sigma Chemical Corp., St. Louis, MO) in Earle's medium was added to the ejaculate in the collection tube. The mixture

was allowed to stand at room temperature for about 5 minutes in order for the semen to liquefy.

Using a dropper, one drop (Male Fertility Kit) or four drops (Male Contraceptive Kit) of the liquefied semen was added to another squeeze bottle (squeeze bottle #2) which had the top removed and contained 150µL (Male Fertility Kit) or 250µL (Male Contraceptive Kit) of a hypotonic solution. Then, one drop (Male Fertility Kit) or 4 drops (Male Contraceptive Kit) of the Rhodamine-123 (Molecular Probes, Inc. Eugene, OR) solution in squeeze bottle #3 was added to squeeze bottle #2 and the top placed back on. The contents were then mixed by tapping with a finger.

Two drops (Male Fertility Kit) or the total content of squeeze bottle #2 was added to a porous filter (about 2.7 µm pore size) mounted on a liquid reservoir and the color visualized. The absence of color was indicative of a concentration of less than about 20 million spermatozoa/mL; faint color was indicative of greater than about 20 million spermatozoa/mL and less than about 40 million spermatozoa/mL; and the presence of color was indicative of greater than about 40 million spermatozoa/mL.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A system for collecting motile sperm from a sperm containing sample, said system comprising:

a) housing means forming a housing having an internal cavity for holding a sperm containing sample; and

b) isolation means, selectively disposable within the cavity and in fluid communication with the sperm containing sample when disposed therein, for isolating motile sperm.

2. The system of claim 1 wherein said isolation means comprises a retainer being selectively and removably positionable within the cavity of the housing and being sized to accomodate the migration of the motile sperm from the sperm sample into an internal passageway formed therethrough, the motile sperm being isolated within the passageway after a sufficient period of time.

3. The system of claim 2 wherein said retainer comprises a fluid that is less dense than the sample.

4. The system of claim 1 wherein said retainer comprises a porous membrane.

5. A system of claim 1, which additionally comprises a means for testing the motile sperm to determine the pregnancy potential of the sample.

6. The system of claim 5 wherein said means for testing comprises a means for determining an indicator of lipid peroxidation or a change in an indicator of lipid peroxidation.

7. The system of claim 6 wherein said means for determining comprises a means for quantitating sperm superoxide dismutase activity using an appropriate superoxide dismutase antibody.

8. The system of claim 7 wherein said superoxide dismutase antibody is colorimetrically labeled.

9. The system of claim 5 wherein said means for testing comprises a means for determining the number of motile sperm within the sperm containing sample.

10. The system of claim ' herein said means for determining comprises a means for quantitating a sperm lipid or a sperm protein.

11. The system of claim 10 wherein the sperm protein is glutathione peroxidase.

12. An assay for determining the pregnancy potential of a sperm containing sample, comprising the steps of:

a) isolating sperm from a sperm containing sample, and

b) testing the sperm to determine the pregnancy potential of the sample.

13. An assay of claim 12 wherein the isolating step a) results in the isolation of mainly motile sperm from the sperm containing sample.

14. An assay of claim 13, wherein prior to the isolating step, an additional step of liquefying the sample is performed.

15. An assay of claim 12, wherein the testing comprises a means selected from the group consisting of quantitating an indicator of lipid peroxidation, quantitating a change in an indicator of lipid peroxidation and quantitating a sperm component sperm.

16. An assay of claim 15, wherein the indicator of lipid peroxidation is selected from the group consisting of a lipid peroxidation breakdown product, sperm phosphatidylethanolamine/phosphatidylcholine ratio and sperm motility.

17. An assay of claim 15, wherein the change in an indicator of lipid peroxidation is selected from the group consisting of: superoxide dismutase activity, oxo-8-deoxyguanosine level, surface superoxide dismutase immunofluorescence, ratio of saturated to unsaturated fatty acids and creatine kinase activity.

18. An assay of claim 15, wherein the sperm component is a sperm protein.

19. An assay of claim 18 wherein the sperm protein is selected from the group consisting of: a flagella protein, a glycolytic enzyme, glutathione peroxidase, a nuclear protein, a mitochondrial protein, an acrosomal protein, α-tubulin, lactate dehydrogenase (LDH-X), a sperm protamine and acrosin.

20. An assay of claim 15, wherein the sperm component is a sperm lipid.

21. An assay of claim 20, wherein the sperm lipid is selected from the group consisting of: phosphatidylglycerol, seminolipid, docosahexaenoic acid, cholesterol, a phospholipid, a glycolipid, a triglyceride, and a fatty acid.

22. An assay for determining the pregnancy potential of a sperm containing sample comprising the steps of:

a) contacting the sperm containing sample with an appropriate amount of a detectable sperm reagent, thereby forming a sperm reagent/sperm complex; and

b) quantitating the sperm reagent/sperm complex, to determine the pregnancy potential of the sperm containing sample.

23. An assay of claim 22, wherein prior to the isolating step, an additional step of liquefying the sample is performed.

24. An assay of claim 22, wherein the sperm reagent is an antibody against a sperm protein.

25. An assay of claim 24, wherein the sperm protein is selected from the group consisting of α-tubulin, lactate dehydrogenase (LDH-X), protamine, acrosin, a flagella protein, a glycolytic enzyme, glutathione peroxidase, a nuclear protein and a mitochondrial protein.

26. An assay of claim 22 wherein the sperm reagent is an antibody against a sperm lipid.

27. An assay of claim 26, wherein the lipid is selected from the group consisting of phosphatidylglycerol, seminolipid, docosahexaenoic acid, cholesterol, a phospholipid, a glycolipid, a triglyceride, and a fatty acid.

28. An assay of claim 22 wherein the sperm reagent is a dye or tracer that interacts with a sperm protein.

29. An assay of claim 22 wherein the sperm reagent is a dye or tracer that interacts with a sperm lipid.

30. An assay of claim 22, wherein the reagent interacts with a sperm antioxidant enzyme or a lipid peroxidation breakdown product.

31. An assay of claim 30, wherein the lipid peroxidation product is selected from the group consisting of oxo-8-deoxyguanosine level, ratio of saturated to unsaturated fatty acids and creatine kinase activity.

32. An assay of claim 22, wherein prior to step b), the sample of step a) is filtered, so that sperm reagent/sperm complex is retained on a filter and uncomplexed sperm reagent passes through the filter.

33. An assay of claim 32, wherein the sperm reagent is colored and the absence of color on the filter is indicative of less than about 20 million spermatozoa/mL and therefore of low pregnancy potential; the presence of a faint color is indicative of greater than about 20 and less than about 40 million spermatozoa/mL and therefore of borderline pregnancy potential; and intense color is indicative of greater than about 40 million spermatozoa/mL and therefore of high pregnancy potential.

34. An assay of claim 33, wherein the sperm reagent is selected from the group consisting of a protein dye and a lipid dye.

35. An assay of claim 22, wherein the sperm containing sample is a seminal plasma-free sperm sample.

36. An assay of claim 35, wherein the sperm containing sample consists only of motile sperm cells.

37. An assay of claim 22 wherein the sperm reagent is an anti-sperm antibody.

38. An assay of claim 37, wherein the anti-sperm antibody is labeled with a detectable particle.

39. An assay of claim 38, wherein the detectable particle is a gold particle or a colored latex particle.

40. An assay of claim 37 wherein the anti-sperm antibody is selected from the group consisting of an anti-human sperm polyclonal antibody and an anti-human glutathione peroxidase antibody.

41. An assay for identifying the pregnancy potential of a sperm containing sample, comprising the steps of:

a) contacting a sperm containing sample with a first detectable particle containing an anti-superoxide dismutase antibody and a second detectable particle containing an anti-glutathione peroxidase antibody thereby forming sperm/ first detectable particle complexes and sperm/ second detectable particle complexes;

b) filtering the sample of step a), so that sperm/ first detectable particle complexes and sperm/second detectable particle complexes are retained on a filter and unbound first detectable particles and second detectable particles pass through the filter; and

c) quantitating each of the first detectable particle and the second detectable particle, wherein a ratio of first detectable particle: second detectable particle below about 1 is indicative of a low pregnancy potential sample; a ratio above about 1 and below about 1.5 is indicative of a borderline pregnancy potential sample; and a ratio above about 1.5 is indicative of a high pregnancy potential sample.

42. An assay of claim 41 wherein the first detectable particle and second detectable particle are distinct and are selected from the group consisting of a gold particle and a colored latex particle.

43. An assay of claim 41 wherein step c), the ratio is determined by quantitating the color resulting from the mixture of the first detectable particle and the second detectable particle.

44. An assay of claim 41 wherein step c), the ratio is determined by comparing the presence of two different colors

45. An assay for determining the pregnancy potential of a sperm containing sample, comprising the steps of:

a) contacting a sperm containing sample with a detectable particle containing anti-sperm antibodies thereby forming sperm/detectable particle complexes;

b) depositing the sample of step a) onto a colored slide that contrasts with the color of the detectable particles; and

c) quantitating the sperm/detectable particle complexes wherein the absence of the detectable particle color is indicative of less than about 20 million spermatozoa/mL and therefore of a low pregnancy potential sample; faint detectable particle color is indicative of equal to or greater than about 20 and less than about 40 million spermatozoa/mL and therefore of a borderline pregnancy potential sample; and the presence of detectable particle color is indicative of greater than about 40 million spermatozoa/mL and therefore of a high pregnancy potential sample.

46. An assay of claim 45, wherein the detectable particle is a latex particle

47. An assay of claim 46 wherein the latex particle is colored.

48. A method for determining the pregnancy potential of a sperm containing sample, comprising the steps of:

a) obtaining multiple sperm containing samples from a donor over time;

b) obtaining an aliquot from each of the multiple sperm containing samples; and

c) testing each aliquot to determine pregnancy potential.

49. A method of claim 48, wherein step c), the pregnancy potential is determined based on determining an indicator of lipid peroxidation or a change in an indicator of lipid peroxidation.

50. A method of claim 49, wherein the indicator of lipid peroxidation is superoxide dismutase activity.

51. A method of claim 48, which additionally comprises the step of: d) using at least one sperm sample identified as having high pregnancy potential in an assisted reproductive technology.

52. A method of claim 51, wherein the assisted reproductive technology is selected from the group consisting of in vitro fertilization, gamete intrafallopian transfer, intrauterine insemination and intracytoplasmic sperm injection.

53. A system for quantitating the relative amounts of at least two analytes in a sample, said system comprising:

a) at least two reagents that specifically bind to each analyte,

b) means for producing a color upon interaction of the reagent with the corresponding analyte and

c) means for measuring the color produced upon mixing of the reagents.

54. A system of claim 53 wherein the reagents are antibodies with bound colored particles.

55. A system of claim 54 wherein the colored particles are latex particles.

56. A system of claim 55 wherein the latex particles have two different colors that produce a distinct third color.

57. A system of claim 56 wherein the preferred color pairs are blue-yellow and red-yellow, so that a 1:1 mixture produces green and orange, respectively.

58. A system of claim 53 wherein the analytes are sperm proteins.

59. A system of claim 58 wherein the sperm proteins are selected from a group consisting of superoxide dismutase and glutathione peroxidase.

60. A system of claim 57 wherein the analytes are sperm lipids.

61. A system of claim 60 wherein the sperm lipids are selected from a group consisting of phosphatidylglycerol, seminolipid, cholesterol and docosahexaenoic acid.

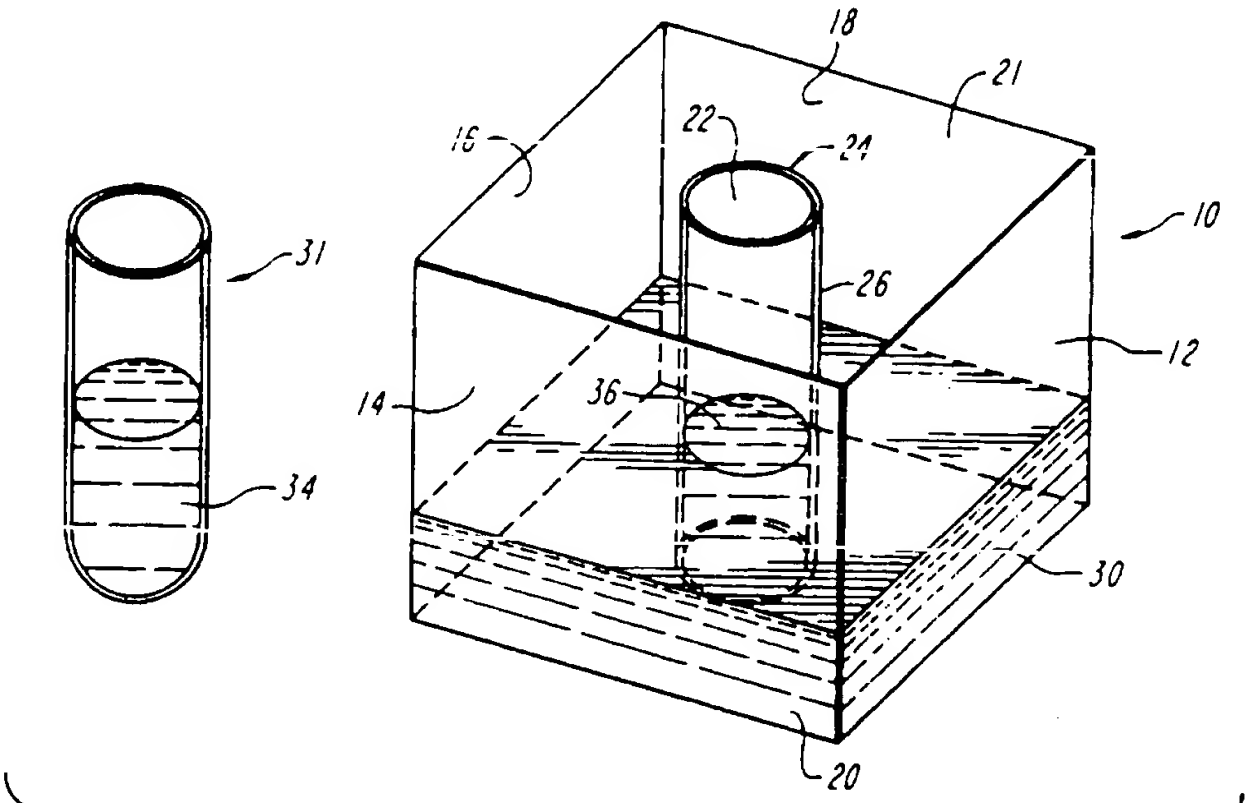

FIG. 1

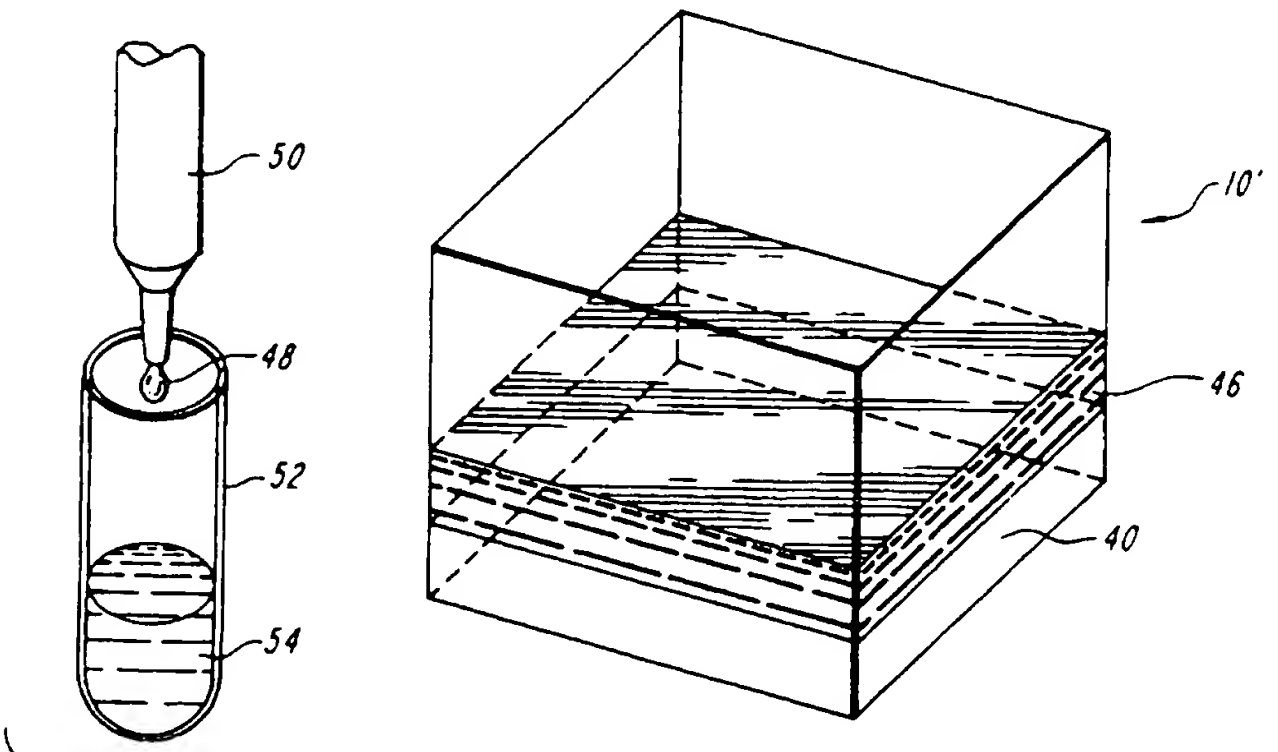

FIG. 2

BNSDOCID <WO___9613225A2_IA>

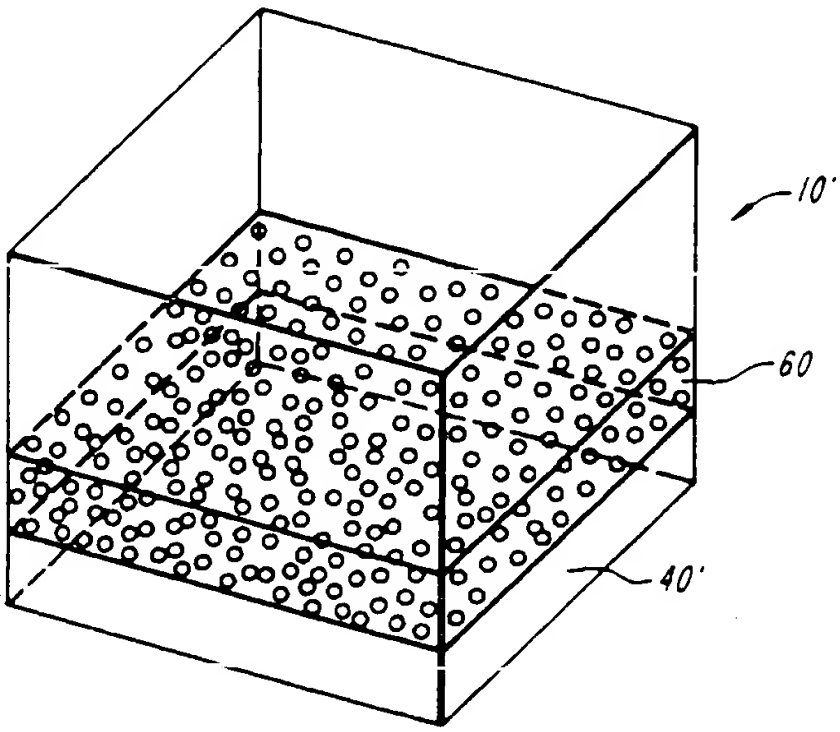

FIG. 3

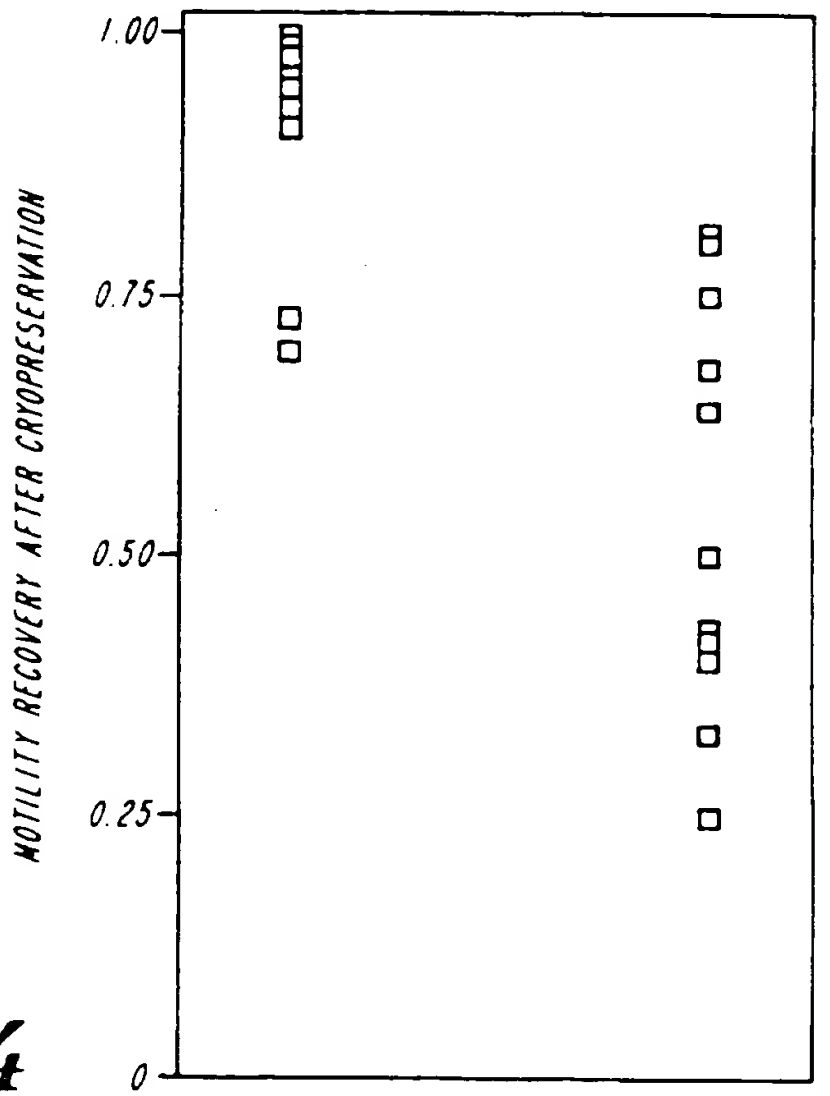

FIG. 4

BNSDOCID: <WO__9613225A2_IA>